The Lability of the Products of Mitochondrial Protein Synthesis in *Saccharomyces cerevisiae*

A NOVEL METHOD FOR PROTEIN HALF-LIFE DETERMINATION

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A method for the determination of the half-life of mitochondrial translation products in yeast *in vivo* is proposed. The method uses inhibitors of cytoplasmic and mitochondrial protein synthesis and is based on double-labelling pulse-chase techniques, the second label being used to estimate 'post-incorporation' during the 'chase'. For the first time the difference between post-incorporation and the widely known recycling of the label is considered. These studies show that, in the turnover of mitochondrial translation products, the problem of post-incorporation into mitochondria (especially from the cell sap) is predominant. The results obtained with this procedure indicate that the half-life of the products of mitochondrial protein synthesis in yeast at the late-exponential phase is about 60 min. The results suggest that mitochondrial translation products are subject to proteolysis to acid-soluble forms.

The increasing interest in the degradation of mitochondrial proteins arises from the physiological role of protein catabolism and especially from the regulatory functions of intramitochondrial proteinases in mitochondrial formation (Gear *et al.*, 1974; Wheeldon *et al.*, 1974; Rajwade *et al.*, 1975). In previous studies the half-life of average mitochondrial protein or crude protein subfractions of rat liver mitochondria was found to be 7–12 days (Fletcher & Sanadi, 1961; Bailey *et al.*, 1967; Beattie *et al.*, 1967; Brunner & Neupert, 1968; Beattie, 1969). These values were later found to be overestimated because of recycling of the radioactive label used. Various attempts were made to overcome this difficulty (see the Discussion section), and half-lives of 4–6 days were observed (Swick *et al.*, 1968; Arias *et al.*, 1969; Gear *et al.*, 1974). Data are available on the renewal rates of several individual mitochondrial protein components such as cytochrome *c* (Druyan *et al.*, 1969; Aschenbrenner *et al.*, 1970; Ip *et al.*, 1974), cytochrome *b* (Druyan *et al.*, 1969) cytochrome oxidase (Druyan *et al.*, 1969; Aschenbrenner *et al.*, 1970), ornithine aminotransferase (Swick *et al.*, 1968; Ip *et al.*, 1974), alanine aminotransferase (Swick *et al.*, 1968) and δ -aminolaevulinate synthetase (Marver *et al.*, 1966).

Of particular interest to the problem of mitochondrial inner-membrane assembly are the results

on specific mitochondrial translation products, which are incorporated into the inner membrane and probably control proper integration of complementary cytoplasmic components (Ebner *et al.*, 1973; Tzagoloff, 1971). Yet information on degradation rates of these polypeptides *in vivo* has been lacking, although the possible role of such degradative processes in regulation of membrane assembly has been extensively discussed by Wheeldon *et al.* (1974) and Gear *et al.* (1974).

Organisms such as *Saccharomyces cerevisiae* and *Neurospora crassa*, which were used to study the assembly of catalytic complexes of the mitochondrial inner membrane, are apparently most suitable for studies on turnover of mitochondrial translation products. In micro-organisms the rates of protein breakdown are usually determined by a pulse-chase technique (see review by Goldberg & Dice, 1974). The chase of the label with the excess of unlabelled amino acid is indeed capable of suppressing recycling of the label. This procedure, however, fails to prevent 'post-incorporation' of the labelled precursor, which is retained within the cell after washing.

A method for protein half-life determination that accounts for post-incorporation is proposed below. This method was specially designed for mitochondrial translation products, where the extent of specific

(cycloheximide-resistant) incorporation is significantly lower than post-incorporation into mitochondria after removal of the cycloheximide block.

Materials and Methods

Chemicals and organism

L-[4,5-³H]Leucine (sp. radioactivity 1 Ci/mmol) and L-[1-¹⁴C]leucine (sp. radioactivity 62 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.; chloramphenicol and cycloheximide were from Sigma Chemical Co., St. Louis, MO, U.S.A.

The strain of *Saccharomyces cerevisiae* yeast wild-type diploid and the conditions of growth have been described previously (Luzikov *et al.*, 1971).

Experimental

The culture was grown in a fermenter until the late-exponential phase, and two batches (1200 ml each) of the yeast suspension were taken. One of them was incubated with [³H]leucine (200 µCi/litre) and cycloheximide (25 mg/litre), the other with [¹⁴C]leucine (100 µCi/litre), cycloheximide (25 mg/litre) and chloramphenicol (4 g/litre). In such conditions, inhibition of the incorporation of the labelled amino acid into mitochondrial proteins was more than 95%. After a 60 min incubation the cells were washed seven times with unlabelled growth medium to remove the protein-synthesis inhibitors and radioactive amino acids. The final pellets were pooled and placed in the unlabelled growth medium corresponding to the late-exponential phase of growth, which contained 500 mg of non-radioactive leucine/litre (20000-fold excess with respect to initial concentration of [³H]leucine). Incubation of the yeast was continued for 3 h under standard conditions, and samples of the suspension were withdrawn, immediately chilled and washed at 2°C.

The efficiency of the washing procedure with respect to removal of chloramphenicol after the pulse was evaluated in a separate experiment where the cells were incubated with chloramphenicol for 60 min, then washed and tested for their ability to incorporate the label into mitochondrial protein in the presence of cycloheximide. Incorporation was similar to that into the control suspension (no preincubation), which indicated that the complete removal of chloramphenicol during washing had taken place. Cycloheximide and chloramphenicol produced no detectable alterations in the yeast cells, as, after the inhibitors were removed, cell number, cell respiration and protein content of mitochondria and postmitochondrial supernatant were comparable with those in the control suspension incubated in a fermenter without cycloheximide or chloramphenicol (Table 1). Immediate and complete recovery from the cycloheximide and chloramphenicol treatment was also observed with exponentially growing cells (results not shown).

Isolation of subcellular fractions and processing of samples

The cell homogenate was prepared as described by Tzagoloff (1969) in a medium containing 0.55 M-mannitol, 100 mM-Tris and 2 mM-MgCl₂, adjusted to pH 7.4 with HCl. The mitochondrial fraction was sedimented quantitatively by centrifugation of the homogenate at 20000g for 30 min. The proteins of mitochondria and the postmitochondrial supernatant were precipitated by trichloroacetic acid (final concn. 10%, w/v) and reprecipitated from 0.5 M-NaOH by the method of Hochberg *et al.* (1972). Then the pellets were incubated in 7% (w/v) trichloroacetic acid at 90°C to hydrolyse the aminoacyl-tRNA complexes and treated as described by Lamb *et al.* (1968) with the following solutions: 7% trichloroacetic acid/aq. 33% (v/v) ethanol, dehydrated acetone, ethanol/ether (3:1, v/v) and ether.

Table 1. Cell number, cell respiration and protein content of subcellular fractions of *Saccharomyces cerevisiae* after labelling in the presence of cycloheximide and chloramphenicol

Experimental conditions are described in the Materials and Methods section. A refers to the control suspension; B refers to the suspension incubated as described in the presence of the inhibitors of protein synthesis, then washed and transferred into a medium containing 500 mg of unlabelled leucine/litre.

Incubation time (h)	10 ⁻⁶ × Cell no. (per ml)		Mitochondrial protein (mg/100 ml of cell suspension)		Protein of postmitochondrial supernatant (mg/100 ml of cell suspension)		Cell respiration (ng-atoms of O/min per mg dry wt. of cells)	
	A	B	A	B	A	B	A	B
0	50.0	50.5	0.76	0.74	28.0	29.5	235	240
1	52.0	52.5	0.79	0.78	28.7	29.0	238	238
2	51.7	52.0	0.81	0.79	30.5	29.5	236	238
3	52.3	51.8	0.80	0.81	30.2	30.0	239	240

Radioactivity counting

The final precipitates (2–4 mg of protein) were dissolved in 1 ml of aq. 25% (v/v) NH_3 by incubation at 50°C for 30 min and transferred into glass scintillation vials containing 10 ml of toluene scintillator solution [4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre of toluene/Triton X-100 (2:1, v/v)]. Samples of the trichloroacetic acid-soluble fraction of mitochondria (0.5 ml) were mixed with 10 ml of dioxan scintillator solution [4 g of 2,5-diphenyloxazole, 200 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 60 g of naphthalene per litre of 20% (v/v) ethanol in dioxan] in glass scintillation vials. Counting was performed in a Nuclear-Chicago Mark I liquid-scintillation spectrometer. To obtain the conditions of highest counting efficiency and minimum sensitivity to quenching, all samples were counted at the 'balance point'. The optimum spectrometer setting for simultaneous ^3H and ^{14}C counting was obtained by using the procedure outlined in the *Mark I Liquid Scintillation Computer* (Nuclear-Chicago Corp., Publication no. 713670, February 1967). The internal standards were L-[1- ^{14}C]leucine (sp. radioactivity 25 mCi/mol) and L-[4,5- ^3H]leucine (sp. radioactivity 250 mCi/mol) from The Radiochemical Centre. Mean efficiencies of 20% for ^3H and 38% for ^{14}C were obtained with the dioxan scintillator; in the toluene scintillation system mean efficiencies were 15% for ^3H and 58% for ^{14}C . Spill-over of ^3H into the ^{14}C channel was 0.2%, and spill-over of ^{14}C into the ^3H channel did not exceed 5% for both scintillators.

Radioactivities of samples were counted at 6°C for 10 min until total counts were at least 50-fold above the background, yielding a relative uncertainty of less than 2%.

Reproducibility

The data presented are the averages of six independent experiments. The final calculated values varied between individual experiments within $\pm 7\%$ of the mean.

Measurement of cell respiration and protein content

The cell respiration was measured polarographically (Mackler *et al.*, 1962). The protein content was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

The dual origin of the proteins of the mitochondrial inner membrane (Ashwell & Work, 1970; Linnane *et al.*, 1972; Schatz & Mason, 1974) makes the existence of a specific labelling procedure the prime requirement for elucidation of the fate of mitochondrial translation products. The intramitochondrial and cytoplasmic protein-synthesizing systems can be distinguished by their sensitivity to

inhibitors such as chloramphenicol and cycloheximide (Linnane *et al.*, 1972; Schatz & Mason, 1974). The only other known alternative to this is the method of Feldman & Mahler (1974), which is based on specific retention of *N*-[^{14}C]formylmethionine in the products of mitochondrial protein synthesis, but some complications, such as multiple ways of [^{14}C]formate incorporation (see, e.g., Schatz & Mason, 1974), make this method only semi-quantitative and thereby inapplicable to turnover studies. So the method proposed here uses the inhibitors of protein synthesis and relies on the following considerations. If yeast culture is pulse-labelled with a radioactive amino acid (^3H]leucine) in the presence of an inhibitor of cytoplasmic protein synthesis (cycloheximide), the label is incorporated exclusively in the mitochondrial membrane proteins of intramitochondrial origin (for review see Schatz & Mason, 1974). Content of these selectively labelled products cannot be followed directly because of post-incorporation. In fact, even thorough washing at the end of the pulse would not prevent a certain amount of the radioactive amino acid from persisting within the cell (see, e.g., Jackl & Sebald, 1975) in specific compartments or bound to tRNA. This residual label will therefore be incorporated into protein after the removal of the cycloheximide block. Thus the content of the selectively labelled products of mitochondrial protein synthesis ($^3\text{H}_{\text{m, synth.}}$) at any moment is described by eqn. (1):

$$^3\text{H}_{\text{m, synth.}} = ^3\text{H}_{\text{m}} - ^3\text{H}_{\text{m, p}} \quad (1)$$

where $^3\text{H}_{\text{m}}$ is the experimentally determined amount of [^3H]leucine in the protein of the mitochondrial fraction, and $^3\text{H}_{\text{m, p}}$ is a complex quantity integrating the extents of post-incorporation into mitochondrial proteins synthesized both in mitochondria and in cytoplasm.

The usual means of preventing recycling of the label used in pulse-chase techniques is isotopic dilution with an excess of the corresponding unlabelled amino acid (Goldberg & Dice, 1974). It also appears to be efficient in suppressing post-incorporation when applied to studies on, for example, degradation of total cell protein, where post-incorporation by itself is negligible in comparison with the gross incorporation of radioactive amino acid during the pulse. As could, however, be expected, the above procedure proved to be insufficient for the purposes of the present study. This peculiarity is undoubtedly due to the fact that the protein synthesized on mitochondrial ribosomes constitutes not more than 2% of the total cell protein (Hawley & Greenawalt, 1970). Hence, even after thorough washing, the residual amount of the labelled precursor retained within the cell will be high compared with the specific incorporation into mitochondrial translation products. Indeed, radio-

activity of the trichloroacetic acid-soluble fraction of the cell sap on incubation of cells with [^3H]leucine reached 4.5×10^6 d.p.m. in our experiments. Repeated washing (see the Materials and Methods section) decreased this value to about 8×10^4 d.p.m., which was less than 2% of the initial concentration; the radioactivity of the acid-soluble fraction of mitochondria was 5500 d.p.m. At the same time, incorporation into the products of mitochondrial protein synthesis amounted only to 400 d.p.m. Thus the amount of residual precursor at the beginning of the chase still exceeds the specific incorporation by two orders of magnitude. Account should be also taken of the substantial amount of the labelled precursor retained in the form of aminoacyl-tRNA. [The cycloheximide block apparently leads to 'saturation' of tRNA with corresponding amino acids (as well as with the labelled one). Subsequent addition of an excess of unlabelled amino acid, however large it may be, provides no competition with these complexes.] Thus it becomes clear that complete suppression of post-incorporation under such conditions is impossible.

In fact, Fig. 1 demonstrates the occurrence of post-incorporation even in the presence of a 20000-fold excess of unlabelled leucine. As follows from the

Fig. 1. Radioactivities of the protein of mitochondrial fraction and postmitochondrial supernatant during the chase period

Yeast suspension was incubated for 60 min with radioactive leucine and inhibitors of protein synthesis (see the Materials and Methods section) and then washed and transferred into a non-radioactive medium containing 500 mg of unlabelled leucine/litre (zero time of the chase). (a) shows changes in relative radioactivities of the two labels in the protein of mitochondrial fraction; (b) shows the same for protein of postmitochondrial supernatant. ●, [^3H]Leucine; ○, [^{14}C]leucine. The data are expressed as percentages of maximal values, which were (d.p.m./sample of suspension): (a) 2300 for ^3H , 1470 for ^{14}C ; (b) 7100 for ^3H , 4530 for ^{14}C .

values given, only about 2–3% of the retained precursor is incorporated into mitochondria during the chase. Nevertheless, even this small post-incorporation produces a significant elevation over the initial radioactivity immediately after the pulse. It should be noted that, since only 5–15% of mitochondrial protein is intramitochondrially synthesized (Sebald *et al.*, 1968; Henson *et al.*, 1968; Schweyen & Kaudewitz, 1970; Hawley & Greenawalt, 1970; Coote & Work, 1971), most of the label post-incorporated would go into mitochondrial components of cytoplasmic origin.

The above signifies that the aims of the present study required application of a method capable of direct estimation of post-incorporation. The method proposed here was developed on the basis of a double-labelling pulse-chase technique used by Sebald *et al.* (1973).

As indicated in the Materials and Methods section, pulse-labelling of the yeast suspension included two parallel incubations: one with [^3H]leucine in the presence of cycloheximide, the other with [^{14}C]leucine in the presence of cycloheximide and chloramphenicol, the latter inhibiting protein synthesis in mitochondria. The pulse was terminated by repeated washing; samples were mixed and transferred into a non-radioactive medium. Because both cytoplasmic and mitochondrial protein-synthesizing systems were blocked during incubation with [^{14}C]leucine, an analogue to eqn. (1) may be written:

$$^{14}\text{C}_{\text{m, synth.}} = ^{14}\text{C}_{\text{m}} - ^{14}\text{C}_{\text{m, p}} = 0 \quad (1a)$$

that is:

$$^{14}\text{C}_{\text{m}} = ^{14}\text{C}_{\text{m, p}}$$

In other words, the content of [^{14}C]leucine in mitochondrial protein at any moment is indicative of the extent of post-incorporation during the chase period. Thus if the proportionality coefficient between the amounts of post-incorporation of [^3H] and [^{14}C]leucine:

$$\alpha = \frac{^3\text{H}_{\text{m, p}}}{^{14}\text{C}_{\text{m, p}}} \quad (2)$$

is found, the value sought ($^3\text{H}_{\text{m, synth.}}$) can be calculated from the experimentally determined quantities $^3\text{H}_{\text{m}}$ and $^{14}\text{C}_{\text{m}}$:

$$^3\text{H}_{\text{m, synth.}} = ^3\text{H}_{\text{m}} - \alpha ^{14}\text{C}_{\text{m}} \quad (3)$$

The metabolic identity of [^3H]leucine and [^{14}C]leucine dictates that α is a constant for any cell fraction at all times. This coefficient can therefore be determined from the radioactivities of the protein of postmitochondrial supernatant, where the experimentally obtained values reflect the incorporation of

[^3H]- and [^{14}C]-leucine at each instant after removal of cycloheximide:

$$\alpha = \frac{{}^3\text{H}_{\text{cyt.}}}{{}^{14}\text{C}_{\text{cyt.}}} \quad (4)$$

Coefficient α can also be inferred from ${}^3\text{H}_m$ and ${}^{14}\text{C}_m$ on the following condition. If, after a sufficiently long incubation, post-incorporation significantly exceeds the radioactivity of the pulse-labelled products, which means that:

$${}^3\text{H}_m \gg {}^3\text{H}_{m,\text{synth.}} \quad (5)$$

eqn. (1) can be transformed into:

$${}^3\text{H}_{m,p} = {}^3\text{H}_m - {}^3\text{H}_{m,\text{synth.}} \approx {}^3\text{H}_m \quad (6)$$

and α in eqn. (2) is approximated by the ratio of measured quantities on the basis of eqns. (1a), (5) and (6):

$$\frac{{}^3\text{H}_m}{{}^{14}\text{C}_m} \approx \frac{{}^3\text{H}_{m,p}}{{}^{14}\text{C}_{m,p}} = \alpha \quad (7)$$

Fig. 2 demonstrates the behaviour of the ratios ${}^3\text{H}_{\text{cyt.}}/{}^{14}\text{C}_{\text{cyt.}}$ and ${}^3\text{H}_m/{}^{14}\text{C}_m$ during incubation of yeast cells in non-radioactive medium (chase period). As predicted, ${}^3\text{H}_{\text{cyt.}}/{}^{14}\text{C}_{\text{cyt.}}$ is constant throughout the chase; this ratio is to be considered as α , being governed simply by the ratio of residual amounts of the two labels in the precursor pool. As for ${}^3\text{H}_m/{}^{14}\text{C}_m$, this ratio gradually decreases, reaching a constant value equal to α when ${}^3\text{H}_{m,\text{synth.}}$ in eqn. (6) becomes a negligible quantity.

It should again be emphasized that α is a constant by its physical sense provided that the conditions of post-incorporation are identical for [^3H]leucine and [^{14}C]leucine. The data of Fig. 1 display the similarity

Fig. 2. ${}^3\text{H}/{}^{14}\text{C}$ ratios for the protein of mitochondrial fraction (○) and postmitochondrial supernatant (●). The values were calculated from the data of Fig. 1.

of post-incorporation patterns of the two labels into the protein of both mitochondria and cell sap, though in the former case similarity is somewhat less apparent at short incubation times because of the initial difference in measured radioactivities, which is due to specific incorporation of [^3H]leucine during the pulse (Fig. 1a). This indicates that chloramphenicol in all probability did not affect the distribution of amino acids in precursor pools, post-incorporation conditions being hence identical on incubation with only cycloheximide or with both cycloheximide and chloramphenicol.

Thus substituting the proportionality coefficient from eqns. (4) or (7) in eqn. (3) permits calculation of ${}^3\text{H}_{m,\text{synth.}}$ for every instant of time. The latter quantity represents the radioactivity of the pulse-labelled products of mitochondrial protein synthesis contained in the mitochondrial fraction at the given moment. The half-life of these products in mitochondria can therefore be estimated at 60 min from the decay of ${}^3\text{H}_{m,\text{synth.}}$ shown on Fig. 3. The results presented pertain to the end of the exponential phase of yeast growth in culture, which corresponds to completion of mitochondrial formation (Yotsuyanagi, 1962; Luzikov *et al.*, 1973).

An analogous approach can be applied to evaluation of the release of the label from mitochondrial translation products into the acid-soluble fraction of mitochondria. Fig. 4(a) demonstrates that during the chase period the amount of [^3H]leucine in the trichloroacetic acid-soluble fraction of mitochondria decreases more slowly than that of [^{14}C]leucine. It is apparent that the changes in the intramitochondrial pools of both labels would be identical but for the supply of [^3H]leucine from the breakdown of the pulse-labelled products of mitochondrial protein synthesis. Indeed, this is the only process affecting the mitochondrial content of acid-

Fig. 3. Decrease in the radioactivity of the pulse-labelled mitochondrial translation products. The values of ${}^3\text{H}_{m,\text{synth.}}$ were calculated from eqn. (3).

Fig. 4. Radioactivities of the acid-soluble fractions of mitochondria and postmitochondrial supernatant

The experimental conditions were as in the legend of Fig. 1. (a) demonstrates the radioactivities of [3H]leucine (○) and [14C]leucine (●) in mitochondria; △ is [3H]leucine and ▲ is [14C]leucine in the postmitochondrial supernatant. The data are expressed as percentages of maximal values, which were (d.p.m./sample of suspension): ○, 5500; ●, 3560; △, 80500; ▲, 52300. (b) The data of (a) are presented as 3H/14C ratios for mitochondria (○) and postmitochondrial supernatant (●).

soluble [3H]leucine that is not paralleled for [14C]leucine. Consequently, the following equation can be written:

$$^3H_{m,degr.} = ^3H_{m,a-s} - \alpha' ^{14}C_{m,a-s} \quad (8)$$

where $^3H_{m,degr.}$ is the radioactivity of the products of the breakdown of mitochondrially synthesized proteins in the acid-soluble fraction of mitochondria at any instant, $^3H_{m,a-s}$ and $^{14}C_{m,a-s}$ are the experimentally determined radioactivities of the two labels in the acid-soluble fraction of mitochondria and α' is the proportionality coefficient. Coefficient α' can be found from the $^3H/^{14}C$ ratio in the acid-soluble fraction of the cytoplasm. Since the cell sap contains no proteins synthesized in the presence of cycloheximide, then:

$$^3H_{cyt.,a-s} = \alpha' ^{14}C_{cyt.,a-s} \quad (9)$$

and

$$\frac{^3H_{cyt.,a-s}}{^{14}C_{cyt.,a-s}} = \alpha' = \text{constant} \quad (10)$$

In fact, the data of Fig. 4(b) show that the $^3H/^{14}C$ ratio in the acid-soluble fraction is constant for the

Fig. 5. Radioactivity released into the acid-soluble fraction of mitochondria owing to breakdown of the pulse-labelled mitochondrial translation products

$^3H_{m,degr.}$ values (●) were calculated from the data of Fig. 4 by eqn. (8). (○) represent the corresponding values ($^3H_{cyt.,degr.}$) for the postmitochondrial supernatant.

cytoplasm and increases for the mitochondria, and at zero time $(^3H/^{14}C)_m = (^3H/^{14}C)_{cyt.} = \alpha'$. Again, α' is determined by the same parameters as the coefficient α in eqn. (3), and these values should be equal, which are in accord with the experimental data (compare Figs. 2 and 4b).

Thus eqn. (8) permits one to calculate the content of [3H]leucine released into the acid-soluble fraction as the result of breakdown of mitochondrial translation products. Fig. 5 demonstrates that $^3H_{m,degr.}$ substantially increases during incubation of the yeast culture. As expected, the analogous value ($^3H_{cyt.,degr.}$) for the acid-soluble fraction of the postmitochondrial supernatant was indistinguishable from zero at any moment (Fig. 5).

The results obtained indicate that the decrease in the content of the products of mitochondrial protein synthesis is the result of their proteolysis to acid-soluble form.

Discussion

The estimation of protein half-lives *in vivo* is recognized as presenting a complex experimental problem. Attention has heretofore been focused on the possible ways to exclude or to account for recycling, i.e. reincorporation of the labelled amino acid released by breakdown of presynthesized protein (Swick *et al.*, 1968; Arias *et al.*, 1969; Druyan *et al.*, 1969; Aschenbrenner *et al.*, 1970; Ip *et al.*, 1974; Humphrey & Davies, 1975; Klar & Halvorson, 1975). Yet practically no methods (with the exception considered below) have hitherto taken into account the process of post-incorporation of the label retained in the precursor pool by the end of the pulse. At very

low extents of specific incorporation (as with mitochondrial translation products) the problem of post-incorporation becomes predominant, but the usual recycling of the label can be effectively suppressed by dilution with an excess of unlabelled amino acid. The latter technique, being universally accepted for micro-organisms (Goldberg & Dice, 1974), was used in the present study.

A method theoretically excluding post-incorporation and recycling was designed by Boudet *et al.* (1975) for measuring total protein turnover in plant tissues, where usual pulse-chase experiments are unsuitable owing to storage of the precursor in the vacuole. The experimental procedure included density labelling of *Lemna minor* (duckweed) amino acids with $^2\text{H}_2\text{O}$ followed by the pulse of ^{14}C -labelled amino acid, which under such conditions was incorporated into 'heavy' proteins. After the plants are transferred to H_2O , post-incorporation and recycling of the label should be into the 'light' newly synthesized proteins. The heavy and light proteins were to be separated by isopycnic centrifugation. Post-incorporation, however, was still observed and complicated the analysis. The method is not suitable for measuring the turnover of individual enzymes or particular protein groups. Moreover, Boudet *et al.* (1975) tend to explain the discrepancy between the results obtained and the previous data (Humphrey & Davies, 1975) by the toxic effects of $^2\text{H}_2\text{O}$ or artifacts produced by gel filtration of the samples before isopycnic centrifugation.

Thus the reason for the development of the method proposed in the present work should now be evident, because no adequate means of estimating post-incorporation have heretofore been available. This approach made it possible to determine a half-life of about 60 min for the products of mitochondrial protein synthesis in yeast (or, strictly speaking, for those labelled during the 60 min pulse). The obtained value is substantially less than the half-lives estimated for the average mitochondrial protein in animals (Ashwell & Work, 1970; Siekevitz, 1972) or the total microbial cell protein (Goldberg & Dice, 1974). Yet it should be borne in mind that the present study concerns only a small protein pool constituting about 10% of the mitochondrial protein (see above); also, a number of individual enzymes (Marver *et al.*, 1966; Swick *et al.*, 1968; Ip *et al.*, 1974) are known to display relatively short half-lives, reaching 72 min for δ -aminolaevulinic synthetase.

A certain analogy to the present data can be found in the work of Wheeldon *et al.* (1974), where it was demonstrated that polypeptides synthesized by isolated rat liver mitochondria possess a half-life of about 20 min. They associated the rapid breakdown of mitochondrial translation products with the action of a specific intramitochondrial proteinase (Wheeldon *et al.*, 1974). Similar results have been

obtained in our laboratory for isolated mitochondria of *Saccharomyces cerevisiae* (Kalnov *et al.*, 1977).

In conclusion, a simple novel method that accounts for post-incorporation of the label (under the conditions of suppressed recycling) is proposed for determination of the half-life of the products of mitochondrial protein synthesis *in vivo*. The short duration of the experiment permits comparative determination of the half-lives of mitochondrial translation products at different phases of culture growth, and consequently at different stages of mitochondrial formation.

References

- Arias, I. M., Doyle, D. & Schimke, R. T. (1969) *J. Biol. Chem.* **244**, 3303–3315
- Aschenbrenner, V., Druyan, R., Albin, R. & Rabinowitz, M. (1970) *Biochem. J.* **119**, 157–160
- Ashwell, M. & Work, T. S. (1970) *Annu. Rev. Biochem.* **39**, 251–290
- Bailey, E., Taylor, C. B. & Bartley, W. (1967) *Biochem. J.* **104**, 1026–1032
- Beattie, D. S. (1969) *Biochem. Biophys. Res. Commun.* **35**, 721–727
- Beattie, D. S., Basford, R. E. & Koritz, S. B. (1967) *J. Biol. Chem.* **242**, 4584–4586
- Boudet, A., Humphrey, T. J. & Davies, D. D. (1975) *Biochem. J.* **152**, 409–416
- Brunner, G. & Neupert, W. (1968) *FEBS Lett.* **1**, 153–155
- Coote, J. L. & Work, T. S. (1971) *Eur. J. Biochem.* **23**, 564–574
- Druyan, R., De Bernard, B. & Rabinowitz, M. (1969) *J. Biol. Chem.* **244**, 5874–5878
- Ebner, E., Mason, T. L. & Schatz, G. (1973) *J. Biol. Chem.* **248**, 5369–5378
- Feldman, F. & Mahler, H. R. (1974) *J. Biol. Chem.* **249**, 3702–3709
- Fletcher, M. J. & Sanadi, D. R. (1961) *Biochim. Biophys. Acta* **51**, 356–360
- Gear, A. R. L., Albert, A. D. & Bednarek, J. M. (1974) *J. Biol. Chem.* **249**, 6495–6504
- Goldberg, A. L. & Dice, J. F. (1974) *Annu. Rev. Biochem.* **43**, 835–870
- Hawley, E. S. & Greenawalt, J. W. (1970) *J. Biol. Chem.* **245**, 3574–3583
- Henson, C. P., Weber, C. N. & Mahler, H. R. (1968) *Biochemistry* **7**, 4431–4444
- Hochberg, A. A., Zahlten, R. N., Stratman, F. W. & Lardy, H. A. (1972) *Biochemistry* **11**, 3143–3149
- Humphrey, T. I. & Davies, D. D. (1975) *Biochem. J.* **148**, 119–127
- Ip, M. M., Chee, P. Y. & Swick, R. W. (1974) *Biochim. Biophys. Acta* **354**, 29–38
- Jackl, G. & Sebald, W. (1975) *Eur. J. Biochem.* **54**, 97–106
- Kalnov, S. L., Serebryakova, N. V., Zubatov, A. S. & Luzikov, V. N. (1977) *Biokhimiya* in the press
- Klar, A. J. S. & Halvorson, H. O. (1975) *J. Bacteriol.* **124**, 863–884
- Lamb, A. J., Clark-Walker, G. D. & Linnane, A. W. (1968) *Biochim. Biophys. Acta* **161**, 415–427
- Linnane, A. W., Haslam, J. M., Lukins, H. B. & Nagley, P. (1972) *Annu. Rev. Biochem.* **26**, 163–198

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **169**, 265–275
- Luzikov, V. N., Zubatov, A. S., Rainina, E. I. & Bakeyeva, L. E. (1971) *Biochim. Biophys. Acta* **245**, 321–334
- Luzikov, V. N., Zubatov, A. S. & Rainina, E. I. (1973) *J. Bioenerg.* **5**, 129–149
- Mackler, B., Collipp, P. J., Duncan, H. M., Rao, N. A. & Huenekens, F. M. (1962) *J. Biol. Chem.* **237**, 2968–2974
- Marver, H. S., Collins, A., Tschudy, D. P. & Rechcigl, M. (1966) *J. Biol. Chem.* **241**, 4323–4329
- Rajwade, M. S., Katyare, S. S., Fatterpaker, P. & Sreenivasan, A. (1975) *Biochem. J.* **152**, 379–387
- Schatz, G. & Mason, T. L. (1974) *Annu. Rev. Biochem.* **43**, 51–87
- Schweyen, R. & Kaudewitz, F. (1970) *Biochem. Biophys. Res. Commun.* **38**, 728–735
- Sebald, W., Bücher, Th., Olbrich, B. & Kaudewitz, F. (1968) *FEBS Lett.* **1**, 235–240
- Sebald, W., Machleidt, W. & Otto, J. (1973) *Eur. J. Biochem.* **38**, 311–324
- Siekevitz, P. (1972) *Annu. Rev. Physiol.* **34**, 117–140
- Swick, R. W., Rexroth, A. K. & Strange, J. L. (1968) *J. Biol. Chem.* **243**, 3581–3587
- Tzagoloff, A. (1969) *J. Biol. Chem.* **244**, 5020–5026
- Tzagoloff, A. (1971) *J. Biol. Chem.* **246**, 3050–3056
- Wheeldon, L. W., Dianoux, A.-Ch., Bof, M. & Vignais, P. V. (1974) *Eur. J. Biochem.* **46**, 189–199
- Yotsuyanagi, Y. (1962) *J. Ultrastruct. Res.* **7**, 121–140