Choice of Precursors for the Measurement of Protein Turnover by the Double-Isotope Method

APPLICATION TO THE STUDY OF MITOCHONDRIAL PROTEINS

By ROWLAND J. BURGESS, JOHN H. WALKER and R. JOHN MAYER
*Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre,
Nottingham NG7 2UH, U.K.*

(Received 15 May 1978)

1. The double-isotope concept [Arias, Doyle & Schimke (1969) *J. Biol. Chem.* **224**, 3303–3315] for the measurement of protein turnover was used to estimate the turnover of proteins in subcellular and submitochondrial fractions prepared from rat liver. 2. Double-isotope experiments with [^3H]leucine as first precursor and [^{14}C]leucine as second precursor were used to measure the turnover rates of proteins in subcellular and submitochondrial fractions. Solvent extraction procedures designed to remove lipids and nucleic acids from trichloroacetic acid precipitates only changed the isotope ratio of the microsomal fraction. It was not possible to measure turnover of proteins in mitochondrial and submitochondrial fractions with these precursors. 3. Double-isotope experiments were designed to minimize first-precursor reutilization by employing $\text{NaH}^{14}\text{CO}_3$. [^3H]Arginine was used as second precursor. The turnover rates of protein in subcellular and submitochondrial fractions was measured. Solvent extraction procedures designed to remove lipids and nucleic acids showed changes in the isotope ratio for all subcellular fractions, especially in microsomal and detergent-soluble mitochondrial fractions. Isotope ratios of precipitates after solvent extraction indicate that, whereas considerable heterogeneity exists for the average rates of protein turnover in subcellular fractions, little heterogeneity is observed in the average rates of protein turnover in submitochondrial fractions.

The double-isotope method of Arias *et al.* (1969) was designed to detect differences in the rates of turnover of protein fractions isolated from liver. The great advantage of the method is that it enables two time points on an isotope decay curve to be determined for a given protein species or fraction in a single animal.

The validity of the method depends on a number of assumptions. The radiolabelled amino acid precursors should not be metabolized, and the experiment should be carried out in steady-state conditions. The loss of label from proteins must follow exponential decay kinetics, and at the time of death all the radiolabelled proteins must be in a process of isotope decay, i.e. the measured isotope ratio should not reflect both the rate of turnover and the rate of translocation of a protein to some subcellular compartment (Arias *et al.*, 1969). The last point may be particularly relevant to mitochondrial proteins, which are synthesized mainly in the cytoplasm but also in the mitochondria (Schatz & Mason, 1974; Hundt & Kadenbach, 1977).

Most experiments with this technique have used [^{14}C]leucine and [^3H]leucine as protein precursors. In single-isotope experiments, radiolabelled leucine has been shown to be a poor precursor for measure-

ment of protein turnover because of isotope reutilization (Poole, 1971; Glass & Doyle, 1972; Tauber & Reutter, 1978), which leads to overestimation of protein half-lives. Furthermore it has been elegantly shown (Poole, 1971; Zak *et al.*, 1977) that reutilization of radiolabelled precursors will significantly decrease the sensitivity of the double-isotope technique, since the maximum specific radioactivities of the majority of proteins with half-lives longer than 3–4 days will be similar. Therefore, to maximize the differences in isotope ratios between proteins of similar half-lives, the time interval between the first and second injections should be long and the animals should be killed as soon as possible after the second injection of radiolabelled amino acid (Poole, 1971; Zak *et al.*, 1977).

However, these considerations, particularly with regard to the second precursor, may prejudice the assumption that radiolabelled proteins are in a process of isotope decay (Arias *et al.*, 1969) and mean that the absolute incorporation of the second precursor into protein species or fractions may be so low as to jeopardize measurement of radioactivity in subsequent analyses.

These considerations and criticisms may be overcome by the use of minimally reutilizable precursors,

which would improve the sensitivity of the double-isotope method by increasing the difference in isotope ratio between proteins of similar half-lives.

In single-isotope experiments much-decreased isotope reutilization has been observed with [*guanidino*- ^{14}C]arginine as protein precursor (Schimke, 1964; Hirsch & Hiatt, 1966; Arias *et al.*, 1969; Scornik, 1972). However, minimal reutilization seems to be achieved when $\text{NaH}^{14}\text{CO}_3$ is used as protein precursor (Swick, 1958; McFarlane, 1963; Swick & Ip, 1974). This precursor is predominantly incorporated into arginine, glutamate and aspartate, and the decrease in the specific radioactivity in arginine isolated from liver protein parallels the decrease in the specific radioactivity of liver protein (Swick & Ip, 1974).

We have compared the use of [^{14}C]leucine and [^3H]leucine with $\text{NaH}^{14}\text{CO}_3$ and [^3H]arginine as precursors in the double-isotope technique in order to measure the turnover of proteins in subcellular and submitochondrial fractions of rat liver. The latter two radioisomers have previously been used together to label cytosol proteins in turnover experiments (Bohley *et al.*, 1972, 1977).

Materials and Methods

Animals

Male Wistar rats (220–260 g) were used. They were obtained from the Joint Animal Breeding Unit, Nottingham School of Agriculture, Sutton Bonington, Leics., U.K.

Materials

All chemicals were A.R. grade and were obtained from BDH Chemicals, Poole, Dorset, U.K., Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., or Fisons, Loughborough, Leics., U.K., except for the following, L-[4,5- ^3H]leucine, L-[U- ^{14}C]leucine, $\text{NaH}^{14}\text{CO}_3$ and L-[5- ^3H]arginine monohydrochloride were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Incorporation of a single precursor into protein

(a) *Incorporation of [^3H]leucine into rat liver mitochondrial protein.* Rats were injected intraperitoneally with L-[4,5- ^3H]leucine (100 μCi) and killed after 1, 2 or 4 h. Mitochondrial fractions were prepared by homogenization of liver in 0.25 M-sucrose (1:3, v/v), centrifugation at 500 g_{av} for 10 min and finally by sedimentation at 9000 g_{av} for 10 min. The mitochondrial pellets were washed and stored in 0.2 M-potassium phosphate, pH 7.

(b) *Incorporation of $\text{NaH}^{14}\text{CO}_3$ into rat liver sub-fractions.* Rats were injected intraperitoneally with

$\text{NaH}^{14}\text{CO}_3$ (250 μCi) and killed after 1, 2, 4 or 6 h. The livers were removed and fractionated as described below.

Incorporation of two precursors into protein

In each double-isotope experiment one test and one control rat was used. For the test animal the first precursor was injected 4 days before administration of the second precursor. At the time of injection of the second precursor into the test rat, the control rat was given both labelled precursors as a single injection. Injections were intraperitoneal and were made at 09:00 h. In the first experiment, each rat was injected with 1 mCi of L-[4,5- ^3H]leucine (1.0 ml) and 125 μCi of L-[U- ^{14}C]leucine (2.5 ml). In the second experiment each rat was injected with 5 mCi of $\text{NaH}^{14}\text{CO}_3$ (1.0 ml) and 500 μCi of L-[5- ^3H]arginine monohydrochloride (0.5 ml). At 4 h after the second injection the rats were killed and the livers were removed and fractionated.

Fractionation procedures

(a) *Subcellular fractionation of rat liver.* Livers were homogenized with a Potter-type homogenizer in 0.25 M-sucrose (1:3, v/v), adjusted to pH 7 with NaHCO_3 . The homogenate was then fractionated by the method of de Duve *et al.* (1955). Fractions were stored at -20°C . The quality of the subcellular fractionation was monitored by assaying the following markers: DNA, succinate dehydrogenase (EC 1.3.99.1), acid phosphatase (EC 3.1.3.2), aryl-esterase (EC 3.1.1.2) and lactate dehydrogenase (EC 1.1.1.27).

(b) *Submitochondrial fractionation.* Mitochondrial fractions were centrifuged at 115000 g_{av} for 10 min, and the pellet was subfractionated. Each pellet was frozen and thawed once, and then osmotically disrupted by resuspension in a volume of water equal to that of the initial mitochondrial fraction. After 30 min the preparation was centrifuged at 115000 g_{av} for 60 min. The supernatant together with the supernatant from the initial centrifugation is called the water-soluble fraction. The pellet was then extracted by resuspension for 15 min at 4°C in 1 M-KCl, containing 50 mM-EDTA (adjusted to pH 7.0 with 1 M-HCl). The volume of solution added was equal to that of the original mitochondrial fraction. The preparation was then centrifuged at 115000 g_{av} for 60 min to give a pellet and a supernatant, which is called the salt-soluble fraction.

The pellet was further extracted, in a manner similar to that previously described, with 200 mM-sodium phosphate, pH 7.0, containing 1% (w/v) Tween 20 for 30 min at 4°C to give a pellet and a supernatant, which is called the Tween-soluble fraction.

The pellet was resuspended by adding a volume of 200mM-potassium phosphate (pH 7.0)/5% (w/v) Triton X-100 equal to 4 times the volume of the pellet. After 2h the suspension was centrifuged at 115000g_{av.} for 60min to give a supernatant called the Triton X-100-soluble fraction. A small residue was discarded. All the fractions were stored at -20°C before use.

Partial purification of cytochrome oxidase

The Triton X-100-soluble fraction was mixed with 3 vol. of water to give a final buffer concentration of 50mM-potassium phosphate, pH 7, containing 1% (w/v) Triton X-100. This diluted fraction was applied to a column (6cm × 2.5cm) of DEAE-cellulose (20ml) previously equilibrated in the same buffer. After sample application the column was washed with 4 column volumes of the starting buffer. The eluted protein is called the non-binding protein fraction. Cytochrome oxidase bound as a green band near the top of the column. This green band was eluted by washing the column with 200mM-potassium phosphate, pH 7, containing 1% (w/v) Triton X-100.

Radioactivity measurement

Two methods of sample preparation were used.

(a) A sample (50–100μl) of each subcellular or submitochondrial fraction was precipitated by addition of 0.5–1.0ml of 10% (w/v) trichloroacetic acid. The precipitate was centrifuged at maximum speed for 10min in a bench centrifuge and then washed twice with 10% trichloroacetic acid containing 10mM-leucine or 10mM-arginine. The final pellet was drained, taken up in 0.5ml of 90% (w/v) formic acid and transferred to a scintillation vial. Each sample tube was washed once with 0.5ml of distilled water, and the washings were added to the vial. Finally 10ml of a scintillant containing Triton X-100 (33%, v/v), xylene (67%, v/v), 2,5-diphenyl-oxazole (0.55%, w/v) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.01%, w/v) was added to each vial.

(b) In some experiments fractions were precipitated with 10% trichloroacetic acid, and subsequently each precipitate was successively washed with 1ml of 10% trichloroacetic acid, 95% (v/v) ethanol, acetone and diethyl ether. Each precipitate was then incubated at 90°C for 15min in 1ml of 10% trichloroacetic acid. The final pellet was subsequently solubilized, and radioactivity measured as described above. All radioactivity measurements were carried out on duplicate or triplicate samples. The results in the Tables represent the means. The maximum errors of the isotope ratios are ±2%.

Radioactivity was measured with a Packard Tri-Carb 3375 spectrometer. Single-isotope radioactivity measurements were corrected for quenching by the

channels-ratio method and dual-isotope measurements by the external standard ratio method. Radioactivities are expressed as d.p.m.

Protein determination

Protein at high concentration (5–60mg/ml) was determined by the method of Gornall *et al.* (1949), modified by the addition of 1% Triton X-100 (w/v, final concentration), and at lower concentrations by the method of Wang & Smith (1975).

Results

Fractionation procedures

Liver homogenates were fractionated by the method of de Duve *et al.* (1955). Marker assays gave the expected relative distribution profiles for the subcellular fractions (results not shown). We designed the submitochondrial fractionation as a functional analytical procedure without regard to mitochondrial morphology in which protein-containing fractions are prepared by differential solubilization. Between 80 and 120mg of mitochondrial protein was used in each fractionation procedure. The protein distribution in the submitochondrial fractions is: water-soluble fraction (approx. 42%), salt-soluble fraction (approx. 11%), Tween 20-soluble fraction (approx. 11%), Triton X-100-soluble fraction (approx. 14%), material that does not bind to DEAE-cellulose (approx. 9%) and material that binds to DEAE-cellulose (approx. 4%).

Incorporation of single precursors into protein

(a) *Incorporation of L-[4,5-³H]leucine into mitochondrial protein.* Fig. 1(a) shows the radioactivity of mitochondrial protein as a function of the time after injection of the animal with L-[4,5-³H]leucine. The specific radioactivity of mitochondrial protein decreases to a constant value between 2 and 3h after injection.

(b) *Incorporation of NaH¹⁴CO₃ into rat liver subcellular fractions.* The incorporation of NaH¹⁴CO₃ into proteins in different subcellular fractions shows a similar profile to that seen with L-[4,5-³H]leucine (Fig. 1b). In contrast with that in homogenate and cytosol, the specific radioactivity of mitochondrial and nuclear protein does not reach an approximately constant value until at least 4h after injection.

Incorporation of two precursors into protein

Isotope ratios in Tables 1 and 2 are for subcellular fractions, and those in Tables 3 and 4 are for submitochondrial fractions. Ratios are shown for trichloroacetic acid-precipitable material before and

Fig. 1. Incorporation of single amino acid precursors into rat liver subcellular fractions

(a) Incorporation of L-[4,5-³H]leucine into rat liver mitochondrial fraction. Rats were injected intraperitoneally with L-[4,5-³H]leucine (100 μCi) and killed after 1, 2 or 4 h. A mitochondrial fraction was then prepared and the precursor incorporation measured. (b) Incorporation of $\text{NaH}^{14}\text{CO}_3$ into rat liver subfractions. Rats were injected intraperitoneally with $\text{NaH}^{14}\text{CO}_3$ (250 μCi) and killed after 1, 2, 4 or 6 h. The livers were removed and fractionated, and the precursor incorporation was measured. ■, Homogenate; □, nuclei; ▲, mitochondria; △, postmitochondrial supernatant; Experimental details are as described in the Materials and Methods section.

after solvent extractions. In one preliminary experiment with radioisomers of leucine (i.e. the same as that shown in Tables 1 and 3) and two preliminary experiments with $\text{NaH}^{14}\text{CO}_3$ and [³H]arginine (i.e. the same as that shown in Tables 2 and 4), similar isotope ratios were obtained for the trichloroacetic

acid-precipitable material. The results reported for the experiments with the improved solvent extraction technique were therefore only carried out once.

(a) *Incorporation of [³H]leucine and [¹⁴C]leucine into proteins of subcellular fractions.* Solvent extraction procedures (Table 1) have relatively little effect on ¹⁴C/³H ratios, giving only a slight increase in the ratios in fractions prepared from the liver of the test animal and no change in ratios in fractions prepared from the liver of the control animal. The largest change in isotope ratio is seen in the microsomal fraction prepared from liver of the test animal.

The isotope ratio for proteins in the mitochondrial fraction prepared from the liver of the test animal is peculiar in that the ratio is less than that for protein in the mitochondrial fraction prepared from the liver of the control animal.

(b) *Incorporation of $\text{NaH}^{14}\text{CO}_3$ and [³H]arginine into proteins of subcellular fractions.* The results in Table 2 show that solvent extraction procedures give an increase in ³H/¹⁴C isotope ratios for precipitates in all subcellular fractions prepared from livers of test and control animals. The largest increase in isotope ratio is again seen for the microsomal fractions in test and control animals. The change in isotope ratio after solvent extraction of precipitates from subcellular fractions prepared from the liver of the control animal is greater than for the precipitates from subcellular fractions prepared from the test animal.

The turnover of protein in the mitochondrial fraction is lower than that of proteins of any other subcellular fraction.

(c) *Incorporation of [³H]leucine and [¹⁴C]leucine into proteins from submitochondrial fractions.* The results in Table 3 show that solvent extraction procedures have relatively little effect on the ¹⁴C/³H ratios. After solvent extraction the isotope ratios of precipitated proteins of submitochondrial fractions from the liver of the test animal is still either equal to or less than the ratio for precipitated proteins in submitochondrial fractions from the liver of the control animal.

(d) *Incorporation of $\text{NaH}^{14}\text{CO}_3$ and [³H]arginine into proteins of submitochondrial fractions.* Solvent extraction procedures (Table 4) give a large increase in the isotope ratio in precipitates from the Tween 20- and Triton X-100-soluble fractions. The largest increase in isotope ratio on solvent extraction is seen with precipitates from the fractions prepared from the liver of the control animal. The isotope ratios for precipitates prepared from detergent-soluble submitochondrial fractions of liver of the control animal before solvent extractions is exceptionally small.

The isotope ratios indicate that there is considerable heterogeneity in the average rates of protein turnover in subcellular fractions from liver (Table 2),

Table 1. Turnover of proteins in subcellular fractions from rat liver, measured by the double-isotope method with [^3H]leucine and [^{14}C]leucine as precursors

L-[4,5- ^3H]Leucine (1 mCi) was injected into the test animal 4 days before a second injection of L-[U- ^{14}C]leucine (125 μCi). A mixture of the same quantities of radioisomers was given as a single injection to a control animal at the time of the second injection into the test animal. The animals were killed 4h after the final injections. Subcellular fractionation and extraction procedures are described in the Materials and Methods section. Incorporation of radioactivity was in the range 3000–17000 d.p.m./mg of protein for ^{14}C and 16×10^3 – 100×10^3 d.p.m./mg of protein for ^3H .

Fraction	Isotope ratios ($^{14}\text{C}/^3\text{H}$)			
	Test animal		Control animal	
	Isotope ratio of precipitated material	Isotope ratio of precipitated protein	Isotope ratio of precipitated material	Isotope ratio of precipitated protein
Homogenate	0.20	0.20	0.16	0.15
Nuclear fraction	0.18	0.20	0.16	0.16
Mitochondrial fraction	0.13	0.13	0.15	0.15
Lysosomal fraction	0.23	0.24	0.15	0.15
Microsomal fraction	0.26	0.29	0.16	0.15
Particle-free supernatant	0.18	0.20	0.15	0.15

Table 2. Turnover of proteins in subcellular fractions from rat liver, measured by the double-isotope method with $\text{NaH}^{14}\text{CO}_3$ and [^3H]arginine as precursors

$\text{NaH}^{14}\text{CO}_3$ (5 mCi) was injected into the test animal 4 days before a second injection of L-[5- ^3H]arginine (500 μCi). A mixture of the same quantities of radioisomers was given as a single injection to a control animal at the time of the second injection into the test animal. Subcellular fractionation and extraction procedures are described in the Materials and Methods section. Incorporation of radioactivity was in the range 1200–16000 d.p.m./mg of protein for ^{14}C and 27×10^3 – 130×10^3 d.p.m./mg of protein for ^3H .

Fraction	Isotope ratios ($^3\text{H}/^{14}\text{C}$)			
	Test animal		Control animal	
	Isotope ratio of precipitated material	Isotope ratio of precipitated protein	Isotope ratio of precipitated material	Isotope ratio of precipitated protein
Homogenate	16.9	17.0	6.8	9.4
Nuclear fraction	17.1	19.2	7.3	9.9
Mitochondrial fraction	10.8	11.1	6.7	9.0
Lysosomal fraction	19.7	22.5	6.4	8.7
Microsomal fraction	21.6	26.2	6.1	9.3
Particle-free supernatant	16.0	16.5	7.9	8.8

but that there is little heterogeneity in the average rates of protein turnover in submitochondrial fractions (Table 4).

Discussion

The results presented in Fig. 1 show a decrease to an approximately constant value of the specific radioactivity of trichloroacetic acid-precipitated material prepared from the homogenate, nuclear fraction, mitochondrial fraction and postmitochondrial fraction of rat liver. Constant specific radioactivities are seen in samples from all fractions labelled with either $\text{NaH}^{14}\text{CO}_3$ or [^3H]leucine by 2h after injection, except for samples prepared from

mitochondria that had been labelled with $\text{NaH}^{14}\text{CO}_3$, where constant specific radioactivity was reached between 2 and 4h after injection. Arias *et al.* (1969) have observed a similar decrease in specific radioactivity for samples of homogenate and of smooth and rough endoplasmic reticulum prepared from rat liver after administration of [^3H]leucine. These authors concluded from their results and those of Higashi & Peters (1963) and Siekevitz *et al.* (1967) that the decrease in specific radioactivity of these microsomal fractions could be accounted for by the cytoplasmic synthesis and secretion of proteins from liver.

It is not clear (Fig. 1) why a similar decrease in specific radioactivity of the nuclear and mitochon-

Table 3. *Turnover of proteins in submitochondrial fractions from rat liver, measured by the double-isotope method with [^3H]-leucine and [^{14}C]-leucine as precursors*

The injection schedule, quantities of radioisomers and killing of animals are as shown in Table 1. Submitochondrial fractionation and extraction procedures are described in the Materials and Methods section. Incorporation of radioactivity was in the range 300–10000 d.p.m./mg of protein for ^{14}C and 16×10^3 – 100×10^3 d.p.m./mg of protein for ^3H .

Fraction	Isotope ratios ($^{14}\text{C}/^3\text{H}$)			
	Test animal		Control animal	
	Isotope ratio of precipitated material	Isotope ratio of precipitated protein	Isotope ratio of precipitated material	Isotope ratio of precipitated protein
Water-soluble fraction	0.15	0.15	0.15	0.15
Salt-soluble fraction	0.13	0.13	0.15	0.16
Tween 20-soluble fraction	0.16	0.15	0.15	0.15
Triton X-100-soluble fraction	0.14	0.12	0.17	0.14
Material not binding to DEAE-cellulose column	0.16	0.13	0.17	0.15
Material binding to DEAE-cellulose column*	0.16	0.13	0.13	0.16

* This fraction contains cytochrome oxidase (approx. 50% of the protein).

Table 4. *Turnover of proteins in submitochondrial fractions from rat liver, measured by the double-isotope method with $\text{NaH}^{14}\text{CO}_3$ and [^3H]-arginine as precursors*

The injection schedule, quantities of radioisomers and killing of animals are as shown in Table 2. Submitochondrial fractionation and extraction procedures are described in the Materials and Methods section. Incorporation of radioactivity was in the range 400–3000 d.p.m./mg of protein for ^{13}C and 3×10^3 – 40×10^3 d.p.m./mg of protein for ^3H . Abbreviation: n.d., not determined.

Fraction	Isotope ratios ($^3\text{H}/^{14}\text{C}$)			
	Test animal		Control animal	
	Isotope ratio of precipitated material	Isotope ratio of precipitated protein	Isotope ratio of precipitated material	Isotope ratio of precipitated protein
Water-soluble fraction, material released on freeze-thawing of mitochondrial fraction	9.7	9.6	8.5	9.6
Water-soluble fraction, material released on water lysis of mitochondrial fraction	11.7	11.6	8.5	8.7
Salt-soluble fraction	12.0	11.6	8.9	10.1
Tween 20-soluble fraction	11.3	12.2	6.8	10.0
Triton X-100-soluble fraction	8.6	11.4	3.0	10.0
Material not binding to DEAE-cellulose column	n.d.	11.8	n.d.	9.8
Material binding to DEAE-cellulose column*	n.d.	11.9	n.d.	9.1

* This fraction contains cytochrome oxidase (approx. 50% of the protein).

drial proteins occurs, although small contamination of the nuclear fraction and of the mitochondrial fraction with microsomal membrane (shown by marker-enzyme distribution, results not shown) may similarly explain the observed decrease in specific radioactivities.

An assumption of Arias *et al.* (1969) in the design of the double-isotope method is that the labelled proteins are in a process of isotopic decay. Several authors (Arias *et al.*, 1969; Poole, 1971; Zak *et al.*, 1977) have pointed out the difficulties raised by the problem of compartmentation of newly synthesized

protein, in that if animals are killed shortly after the second injection the double-isotope ratio will give a measure, not of degradation rates, but of a combination of the degradation rate and the time required for translocation of newly synthesized protein to some subcellular compartments. Arias *et al.* (1969) overcame this problem by choosing a time interval after the second injection at which the specific radioactivity of microsomal protein was constant. Similarly we have chosen a period of 4h in our experiments. Paradoxically, for the double-isotope technique with reutilizable precursors and liver proteins of average half-life ($t_{1/2}$ 3–6 days), this minimizes the sensitivity of the method (Poole, 1971; Zak *et al.*, 1977). This is one reason why we have developed a procedure that utilizes minimally reutilizable precursors.

Finally, killing the animal shortly (e.g. 30min) after the final injections of radioisomers (Poole, 1971; Zak *et al.*, 1977) leads to minimal incorporation of the second precursor into liver proteins. This means that the analysis of radioactivity in specific proteins derived from different subcellular compartments becomes very difficult. This problem has been found to be acute for submitochondrial fractions and in the analysis of the subunits of proteins from different subcellular fractions by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Walker *et al.*, 1978).

Use of leucine radioisomers in the double-isotope technique

The effect of solvent extraction on the isotope ratio of trichloroacetic acid-precipitable material prepared from each subcellular fraction was examined (Tables 1 and 3). This is necessary, since the isotope incorporation should be into protein and should not include incorporation into non-protein components that might be precipitated by trichloroacetic acid (Arias *et al.*, 1969). Solvent extraction produced little change in isotope ratios, except for that in the microsomal fraction which may be due to incorporation of radiolabel from leucine into lipid (Stillway *et al.*, 1977).

Isotope ratios for proteins in the mitochondrial fraction (Table 1) prepared from the test animal are lower than in proteins from mitochondrial fractions prepared from liver of the control animal. Similarly, isotope ratios for proteins in the submitochondrial fractions prepared from the test animal (Table 3) are equal to or less than ratios in proteins from submitochondrial fractions prepared from liver of the control animal.

These observations serve to emphasize the inadequacy of radioisomers of leucine in double-isotope experiments, since the ratios indicate little or

no apparent turnover of mitochondria or mitochondrial proteins. The results presented here for mitochondrial proteins reflect the problems discussed above of isotope reutilization and compartmentation of newly synthesized proteins when using the double-isotope technique. The similarity of ratios in mitochondrial proteins from the livers of test and control animals may reflect enhanced reutilization of [^3H]-leucine (injected 4 days before killing) due to the selective retention of [^3H]leucine in a mitochondrial amino acid pool (Portugal *et al.*, 1970) that is used in mitochondrial protein synthesis.

However, the solution to this problem depends on the regulation of the transport of amino acids into mitochondria and on resolution of the contentions that mitochondria may (Alberti & Bartley, 1969; Bartley & Birt, 1970) or may not (Ferdinand *et al.*, 1973; Rubio & Grisolia, 1977) have an intrinsic proteolytic system.

The results shown in Tables 1 and 2 clearly show that the radioisomers of leucine are of little use in the measurement of the turnover of mitochondrial proteins. We therefore decided to use $\text{NaH}^{14}\text{CO}_3$ and [^3H]arginine as the first and second precursors respectively in the double-isotope technique.

Use of $\text{NaH}^{14}\text{CO}_3$ and [^3H]arginine in the double-isotope technique

Solvent extraction of trichloroacetic acid-precipitable material of subcellular and submitochondrial fractions is a prerequisite for the use of these precursors. The results (Table 2 and 4) indicate that a significant incorporation of $\text{NaH}^{14}\text{CO}_3$ into trichloroacetic acid-precipitable non-protein material, probably lipid, must occur. This is further substantiated by the observation that increases in isotope ratio are greatest for subcellular (microsomal) and submitochondrial (detergent-soluble) fractions, which might be expected to contain most lipid. In mitochondrial fractions the incorporation must be into rapidly turning over lipid, since the largest increase in isotope ratio occurs in precipitates from detergent-soluble submitochondrial fractions (Table 4) isolated from the liver of the control animal. If the larger difference in isotope ratios on solvent extraction were due to removal of haem, then similar increases in isotope ratio would be expected from the livers of test and control animals, since haem and protein turnover is likely to be at similarly low rates ($t_{1/2}$ 4–6 days) (Ip *et al.*, 1974; Druyan *et al.*, 1969).

The results in Table 2 show that the isotope ratios for proteins are considerably different in different subcellular fractions, indicating different rates of protein turnover in these fractions. Theoretically the isotope ratios for proteins prepared from sub-

cellular fractions of the livers of control animals should be the same. This is generally observed.

The results in Table 4 indicate that proteins in the submitochondrial fractions turn over at different but similar rates. However, the isotope ratios for proteins in these fractions represent values derived from the average rate of turnover of proteins in each fraction. Therefore the turnover rate of individual proteins can only be measured after isolation of the proteins or their subunits (Walker *et al.*, 1978).

Nicoletti *et al.* (1977) have used leucine radioisomers as precursors for the double-isotope method and have utilized the method of Glass & Doyle (1972) to compute degradation rates (K_D) for proteins in subcellular fractions and mitochondria. Their results indicated that individual enzymes turn over at rates that are similar to (carbamoyl phosphate synthase) or different from (malate dehydrogenase, glutamate dehydrogenase) the average rate of mitochondrial turnover.

In general, the isotope ratios (Table 4) indicate similar average rates of turnover of proteins in different submitochondrial fractions. However, measurements of the turnover of protein subunits resolved by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Walker *et al.*, 1978) indicate that some proteins in a number of submitochondrial fractions turn over at considerably different rates from the average rate of turnover of proteins in that fraction.

We are grateful to Miss E. Edwards for skilled technical assistance. J. H. W. was supported by a Research Studentship from the Science Research Council. R. J. B. is supported by a grant from the Medical Research Council.

References

- Alberti, K. G. M. M. & Bartley, W. (1969) *Biochem. J.* **111**, 763–776
- Arias, I. M., Doyle, D. & Schimke, R. T. (1969) *J. Biol. Chem.* **244**, 3303–3315
- Bartley, W. & Birt, L. M., (1970) in *Essays in Cell Metabolism* (Bartley, W., Kornberg, H. L. & Quayle, J. R., eds.), pp. 1–43, John Wiley, New York
- Bohley, P., Miehle, M., Miehle, C., Kirschke, H., Langner, J., Wiederanders, B. & Ansorge, S. (1972) *Acta Biol. Med. Germ.* **28**, 223
- Bohley, P., Kirsche, H., Langner, J., Riemann, S., Wiederanders, B., Ansorge, S. & Hanson, H. (1977) in *Intracellular Protein Catabolism II* (Turk, V. & Marks, N., eds.), pp. 108–110, Plenum Press, New York and London
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Druyan, R., deBarnard, B. & Rabinowitz, M. (1969) *J. Biol. Chem.* **244**, 5874–5878
- Ferdinand, W., Bartley, W. & Broomhead, V. (1973) *Biochem. J.* **134**, 431–436
- Glass, R. D. & Doyle, D. (1972) *J. Biol. Chem.* **247**, 5234–5242
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Higashi, T. & Peters, T., Jr. (1963) *J. Biol. Chem.* **238**, 3952–3954
- Hirsch, C. A. & Hiatt, H. H. (1966) *J. Biol. Chem.* **241**, 5936–5940
- Hundt, E. & Kadenbach, B. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 1309–1314
- Ip, M. M., Chee, P. V. & Swick, R. W. (1974) *Biochim. Biophys. Acta* **354**, 29–38
- McFarlane, A. S. (1963) *Biochem. J.* **89**, 277–290
- Nicoletti, M., Guerri, C. & Grisolia, S. (1977) *Eur. J. Biochem.* **75**, 583–592
- Poole, B. (1971) *J. Biol. Chem.* **246**, 6587–6591
- Portugal, F. H., Elwyn, D. H. & Jeffay, H. (1970) *Biochim. Biophys. Acta* **215**, 339–347
- Rubio, V. & Grisolia, S. (1977) *FEBS Lett.* **75**, 281–284
- Schatz, G. & Mason, T. L. (1974) *Annu. Rev. Biochem.* **43**, 51–87
- Schimke, R. T., (1964) *J. Biol. Chem.* **239**, 3808–3817
- Scornik, O. A. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1063–1066
- Siekevitz, P., Palade, G. E., Dalner, G., Ohad, I. & Omura, T. (1967) in *Organisational Biosynthesis* (Voegl, H. J., Lampen, J. O. & Bryson, V. eds.), p. 331, Academic Press, New York
- Stillway, L. W., Weigand, D. A., Riefler, J. F. & Buse, M. G. (1977) *Lipids* **12**, 1012–1016
- Swick, R. W. (1958) *J. Biol. Chem.* **231**, 751–764
- Swick, R. W. & Ip, M. M. (1974) *J. Biol. Chem.* **249**, 6836–6841
- Tauber, R. & Reutter, W. (1978) *Eur. J. Biochem.* **83**, 37–45
- Walker, J. H., Burgess, R. J. & Mayer, R. J. (1978) *Biochem. J.* **176**, 927–932
- Wang, C. & Smith, R. L. (1975) *Anal. Biochem.* **63**, 414–417
- Zak, R., Martin, A. F., Prior, G. & Rabinowitz, M. (1977) *J. Biol. Chem.* **252**, 3430–3435