Protein degradation in rat liver during post-natal development

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Protein degradation rates for liver subcellular and submitochondrial fractions from neonatal (8-day), weanling (25-day) and adult rats were estimated by the double-isotope method with $\text{NaH}^{14}\text{CO}_3$ and $[^3\text{H}]\text{arginine}$ as the radiolabelled precursors [Dice, Walker, Byrne & Cardiel (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2093–2097]. Decreased protein degradation rates were found during post-natal development for homogenate, nuclear, mitochondrial, lysosomal and microsomal proteins. A decrease in degradation rates for the immunisolated subunits of monoamine oxidase and pyruvate dehydrogenase was also observed in neonatal and weanling rats respectively. The results suggest coordinate degradation of the subunits of the multi-subunit enzyme pyruvate dehydrogenase. Pyruvate dehydrogenase has a faster rate of degradation in adult rat liver than does cytochrome oxidase. Data analysis suggests heterogeneity of protein degradation rates in the mitochondrial outer membrane and intermembrane space fractions at each developmental stage but not in the mitochondrial inner membrane or matrix fractions. Results obtained for protein degradation rates in adult rat liver by the method of Burgess, Walker & Mayer [(1978) *Biochem. J.* **176**, 919–926] in general confirmed the results obtained for the adult rat liver by the above method. No evidence of a subunit-size relationship for protein degradation was found for proteins in any subcellular or submitochondrial fraction.

Rapid accumulation of proteins during post-natal development (Miller, 1969) must be the result of a controlled imbalance between the processes of protein synthesis and degradation (Mayer, 1979). How this imbalance is created or controlled in tissues is not known. Accumulation of total liver proteins appears to be achieved during post-natal development by a high synthetic rate and a rate of degradation approximately half that in the adult (Conde & Scornik, 1977). This is in contrast with the situation in muscle and brain, where general and individual protein accumulation is thought to take place when both synthesis and degradation rates are declining from an initially high value (Millward *et al.*, 1975; Dunlop *et al.*, 1978; Mayer *et al.*, 1980), although Dahl & Weibel (1979) challenge this conclusion for brain.

It is not known whether the observed decrease in degradation rate of total liver protein is found for all proteins or whether there are differential or selective changes in degradative rates for individual proteins

Abbreviations used: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; IgG, immunoglobulin G.

or groups of proteins from morphologically defined organelle compartments.

Several studies have emphasized both the heterogeneity of protein degradation rates in mitochondrial preparations from adult rat liver (Brunner & Neupert, 1968; Swick *et al.*, 1968; Druyan *et al.*, 1969; Gross *et al.*, 1969; de Bernard *et al.*, 1969; Aschenbrenner *et al.*, 1970; Ashwell & Work, 1970; Ip *et al.*, 1974; Satav *et al.*, 1976; Nicoletti *et al.*, 1977; Walker *et al.*, 1978) and the similarity of degradation rates in at least one mitochondrial subcompartment, the inner membrane (Druyan *et al.*, 1969; Gross *et al.*, 1969; de Bernard *et al.*, 1969; Aschenbrenner *et al.*, 1970). These data may imply selective control of the degradation of proteins or protein groups in mitochondrial subcompartments. However, the real extent of heterogeneity of protein degradation rates in the mitochondrion is currently unknown.

Studies on the changes in mitochondrial enzyme activities in regenerating rat liver led Gear (1970) to propose the unit turnover of proteins of the mitochondrial inner membrane, matrix, intermembrane space and a 'primary' outer membrane.

The supposition of a 'primary' outer membrane was necessary, since several outer membrane proteins are degraded at a higher rate than those of the mitochondrial inner membrane (Brunner & Neupert, 1968; de Bernard *et al.*, 1969; Druyan *et al.*, 1969). This idea is supported by measurements on the turnover of mitochondrial proteins in steady state conditions (Swick *et al.*, 1968).

The double-isotope method was designed to enable the comparison of degradation rates for many proteins from one source (Arias *et al.*, 1969) and may therefore be suited to determine the generality of the decrease in liver protein degradation during development (Conde & Scornik, 1977). However, an essential requirement of the method is that both radiolabelled precursors are incorporated into proteins under identical conditions of protein synthesis. We have overcome this problem in developing tissues with the method of Dice *et al.* (1978) in which protein mixtures from different animals are pooled to enable the double-isotope method to be used in the study of protein degradation in non-steady state conditions. We have also used the double-isotope technique of Burgess *et al.* (1978) to determine the relative rates of protein degradation in mitochondrial subcompartments from adult rat liver. In both sets of experiments precursor reutilization was minimized by using $\text{NaH}^{14}\text{CO}_3$ as the first precursor and $[^3\text{H}]\text{arginine}$ as the second precursor (Burgess *et al.*, 1978). The double-isotope technique and the 4 day time interval between precursor administration were chosen to give the most sensitive measure of relative rates of degradation of mitochondrial proteins (Poole, 1971; Zak *et al.*, 1979). Mitochondrial protein half-lives estimated with non-reutilizable precursors range from 0.7 to 6.1 days (Swick *et al.*, 1968; Druyan *et al.*, 1969; Ip *et al.*, 1974). We have analysed subcellular- and submitochondrial-fraction proteins from adult, weanling and neonatal rat liver by polyacrylamide-gel electrophoresis in the presence of SDS to obtain an estimate of the relative rates of protein degradation. Results have also been obtained for the relative degradation rates of the subunits of pyruvate dehydrogenase, monoamine oxidase and cytochrome oxidase. The results have been analysed to determine the mean half-life of protein mixtures or of isolated protein subunits and to give an estimate of the degree of heterogeneity of half-lives within protein mixtures.

Materials and methods

Animals

Male Wistar rats of three age groups (adult, 220–260 g; weanling, 25-day-old and neonate, 8-day-old) were used. Sheep and rats were obtained from the Joint Animal Breeding Unit, Nottingham

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Materials

ATP, AMP, phosphoenolpyruvate, NADH, NAD^+ , pyruvate kinase, lactate dehydrogenase, cytochrome *c* and aldehyde dehydrogenase were obtained from Boehringer-Mannheim (London), London W.5, U.K. $\text{NaH}^{14}\text{CO}_3$, L-[5- ^3H]arginine monohydrochloride and [2- ^{14}C]tyramine were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were A. R. grade or of the highest quality available 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.

Radiolabelled precursor injection schedule

Developmental studies. Each test and control group of rats was divided into two equal sets of rats (set 1 and 2); set 1 was injected with L-[5- ^3H]arginine and set 2 was injected with $\text{NaH}^{14}\text{CO}_3$. The test group rats that received $\text{NaH}^{14}\text{CO}_3$ were killed 4 days after administration of precursor. All other rats, including control rats, were killed 4 h after administration of precursor.

Each test and control group of adult rats consisted of two rats that received either 1 mCi of L-[5- ^3H]arginine (1 ml) or 4.3 mCi of $\text{NaH}^{14}\text{CO}_3$ (2.8 ml). Each test and control group of weanling rats (25-day-old) consisted of eight rats that were injected with either 0.208 mCi of L-[5- ^3H]arginine (0.21 ml) or 2.39 mCi of $\text{NaH}^{14}\text{CO}_3$ (0.52 ml). A second control group of weanling rats were used that were injected 4 days later than the first control group (i.e. at 29 days) with the same quantity of radio-labelled precursors and were killed 4 h later. Each test and control group of neonates (8-day-old) consisted of 16 rats that were injected with either 0.49 mCi of $\text{NaH}^{14}\text{CO}_3$ (0.1 ml) or 0.1 mCi of L-[5- ^3H]arginine (0.1 ml).

Adult study. In each experiment, one test and one control rat was used. Experiments were performed in duplicate. The test animals received injections of the first precursor 4 days before administration of the second precursor. Each rat was injected with 12.5 mCi of $\text{NaH}^{14}\text{CO}_3$ (1.5 ml) and 1.0 mCi of L-[5- ^3H]arginine (1.0 ml). The rats were killed 4 h after the second injection and the livers were removed and fractionated. Control animals received both precursors simultaneously and were killed 4 h later. In both sets of experiments injections were intraperitoneal and were made at 09:00 h.

Adult and weanling rats were maintained at 22°C and fed *ad libitum* before and after injection of radiolabelled precursors. Expired air from adult and weanling rats was drawn through $\text{Ba}(\text{OH})_2$ during the 4 h immediately after injection of radiolabelled

precursors to prevent gross atmospheric contamination with $^{14}\text{CO}_2$. Neonatal rats were returned to their dams immediately after administration of radiolabelled precursors.

Before removal, all livers were perfused with 0.15 M-NaCl at 0°C. Liver and body weights were recorded for each set of rats.

Fractionation procedures

Subcellular fractionation of rat liver. The method of subcellular fractionation has been described in Burgess *et al.* (1978).

The fractionation was monitored by assaying the following markers: DNA, succinate dehydrogenase (EC 1.3.99.1), acid phosphatase (EC 3.1.3.2), arylesterase (EC 3.1.1.2) and lactate dehydrogenase (EC 1.1.1.27).

Submitochondrial fractionation. Mitochondrial pellets were taken up in isolation medium (70 mM-sucrose / 220 mM-D-mannitol / 2.0 mM-Hepes / 0.5 mg of bovine serum albumin/ml, pH 7.4) to give 100 mg of mitochondrial protein/ml. Phenylmethane-sulphonyl fluoride [10% (w/v) in 95% (v/v) ethanol] was added to give a final concentration of 2 mM. Mitochondria were subfractionated by a modification of the method of Schnaitman & Greenawalt (1968). The mitochondrial suspension was stirred at 0°C for 15 min with an equal volume of digitonin solution (13 g/l). The suspension was then diluted with 3 vol. of precooled (0°C) isolation medium and centrifuged for $10^5 g_{av}$ -min at 4°C. The supernatant was removed; it contains the outer mitochondrial membrane and intermembrane-space proteins. The sedimented mitoplasts were washed by resuspension in isolation medium. The volume was adjusted to one-half of the volume of the diluted digitonin-treated mitochondrial sample and the suspension was recentrifuged. The supernatant was added to the outer membrane and intermembrane space fraction and the combined mixture was centrifuged for $2 \times 10^7 g_{av}$ -min. The supernatant was used as the intermembrane space fraction. The pellet was resuspended in potassium cholate, pH 7.4 (80 g/l) to yield the outer membrane fraction.

The washed mitoplast pellet was suspended in 20 mM-potassium phosphate, pH 7.4 (10 ml per 200 mg of original mitochondrial protein) and freeze-thawed three times with liquid N_2 . The suspension was then centrifuged for $10^7 g_{av}$ -min at 4°C. The supernatant, containing some matrix proteins, was removed and the pellet containing the inner membrane and remaining matrix proteins was solubilized in potassium cholate solution, pH 7.4 (80 g/l), to give the inner membrane fraction. All fractions were stored at -20°C before use.

The quality of the submitochondrial fractionation was monitored by assaying the following marker enzymes: monoamine oxidase (EC 1.4.3.4),

adenylate kinase (EC 2.7.4.3), cytochrome oxidase (EC 1.9.3.1) and malate dehydrogenase (EC 1.1.1.37).

Developmental studies. Livers from each set of rats were pooled and subfractionated as above. The ratio of organelle protein yield for test and control animals from set 1 and set 2 differed slightly ($\pm 4\%$) from the ratio of weights of set 1/set 2 livers. For consistency organelle proteins were always mixed in the ratio of liver weights. Submitochondrial fractionation was performed on each mitochondrial fraction (test sets 1 and 2, control sets 1 and 2) from all age groups and the additional second control sets 1 and 2 from the weanling age group.

Immunoabsorbent chromatography

Monoamine oxidase, pyruvate dehydrogenase and cytochrome oxidase were purified from mitochondrial subfractions by immunoabsorbent chromatography.

Monoamine oxidase. Monoamine oxidase from rat liver was purified by a modification of the method of Dennick & Mayer (1977). A final purification step by gel electrophoresis [10% (w/v) polyacrylamide] in the presence of SDS was included. The subunit of monoamine oxidase was identified after staining with Coomassie Brilliant Blue R and the portion of polyacrylamide gel containing the subunit was excised and homogenized in 20 mM-sodium phosphate buffer, pH 7.4. The purified enzyme (500 μg) was suspended in 16 ml of the buffer. Part (4 ml) of this suspension was mixed with 4 ml of Freund's complete adjuvant and used to inject a sheep at eight sites (four subcutaneous and four intramuscular, 1 ml injections). The remaining gel suspension was mixed with an equal volume of Freund's incomplete adjuvant and injected at 14-day intervals on three occasions with the same injection procedure as described previously. At 10 weeks after the first injection the animal was bled by jugular cannulation and IgG was prepared from the antiserum by the method of Mayer & Walker (1978). Anti-(monoamine oxidase) serum was adsorbed to mitochondria as described in Russell *et al.* (1979) and used to prepare an immunoabsorbent column (Walker & Mayer, 1977). The binding capacity of the immunoabsorbent for monoamine oxidase activity was determined to be $4.8 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{ml of Sepharose})^{-1}$. An equivalent control IgG-Sepharose preparation bound $0.2 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{ml of Sepharose})^{-1}$ of monoamine oxidase activity.

Pyruvate dehydrogenase. Anti-(rat heart pyruvate dehydrogenase) serum was raised in sheep as described in Sinnett-Smith *et al.* (1980). IgG was prepared from the antiserum. The IgG was further purified by immunoaffinity chromatography with pig heart pyruvate dehydrogenase linked to Sepharose

4B. Monospecific IgG eluted from the column with 0.1 M-glycine/HCl, pH 2.8, was used to prepare an immunoadsorbent column with a pyruvate dehydrogenase binding capacity of $20 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{ml of Sepharose})^{-1}$ as determined by the method of Walker & Mayer (1977).

Cytochrome oxidase. The method of preparation of anti-(cytochrome oxidase) serum and coupling to Sepharose 4B is given by Burgess *et al.* (1978). The binding capacity of the prepared immunoadsorbent was $67 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{ml of Sepharose})^{-1}$.

Control IgG was prepared from blood taken from the animals before immunization with enzyme and was used to prepare control IgG-Sepharose adsorbents for each immunoisolation scheme.

Control IgG was bound to Sepharose 4B at the same protein concentration as that used for the anti-(pyruvate dehydrogenase) and anti-(cytochrome oxidase) immunoadsorbents respectively.

Use of immunoadsorbents

Each preparation of the relevant enzyme was divided into two equal samples. One sample was incubated with appropriate immunoadsorbent with a 1.5-fold excess of antibody over enzyme activity. The other half of the preparation was incubated with a volume of control IgG-Sepharose equal to the volume of immunoadsorbent. Incubations were left at 4°C overnight. Unbound protein was eluted with 3 column vol. of potassium cholate, pH 7.2 (80 g/l). Columns were washed with 5 column vol. of 20 mM-potassium phosphate, pH 7.0, containing 0.15 M-NaCl before elution of the enzyme with 3 column vol. of 8 M-urea containing SDS (20 g/l). The eluates were vacuum dialysed against water until the volumes had been decreased to approx. 0.1 ml.

Purification scheme for cytochrome oxidase

Cytochrome oxidase was purified from mitochondrial inner membrane preparations obtained during the developmental experiments by the method of Höchli & Hackenbrock (1978) with the following modifications: (a) the starting material for the preparation was the isolated inner membrane preparation; (b) the Triton X-114 solubilization step was omitted and the mitochondrial membrane preparation was solubilized directly in 100 mM-potassium phosphate, pH 7.4, containing 4% (w/v) sodium cholate; (c) the $(\text{NH}_4)_2\text{SO}_4$ fractionation was carried out as described until the final precipitation steps, which were performed in 100 mM-potassium phosphate, pH 7.4, containing 1.5% (w/v) sodium cholate. After two such 37% $(\text{NH}_4)_4\text{SO}_4$ precipitations the final precipitate was solubilized in sample buffer (0.2 ml) for electrophoresis and then was dialysed overnight against 100 ml of sample buffer.

Polyacrylamide-gel electrophoresis

Slab gels (0.3 cm × 11 cm × 14 cm) containing 2% (w/v) SDS and 8 M-urea were prepared by a modification of the method of Swank & Munkres (1971). The final acrylamide concentration was 10% (w/v) and the acrylamide/bisacrylamide ratio was 37:1.

Preparation of samples for electrophoresis

Samples containing 3–5 mg of protein were dialysed overnight against sample buffer [8 M-urea/2% (w/v) SDS/5 mM-dithiothreitol/0.01 M- H_3PO_4 , finally adjusted to pH 6.8 with Tris] at room temperature.

Electrophoresis procedure

Each sample (0.1–0.2 ml) was mixed with 0.1 ml of glycerol containing 0.003% (w/v) Bromophenol Blue. The mixture was pipetted onto a 3 cm wide sample well in the gel surface. Electrophoresis was carried out at 40 mA per gel until a fluorescent cytochrome *c* protein marker (made by the method of Rinderknecht, 1962) had travelled 80% of the gel length. Bovine serum albumin, ovalbumin and cytochrome *c* were used as protein standards.

Staining of gels

Protein was detected on the gels by a rapid fixing, staining and destaining procedure. The gel was immersed in methanol/acetic acid/water (5:1:5, by vol.) for 1 h, removed and stained for 30 min with a solution of 0.1% (w/v) Coomassie Brilliant Blue in methanol/acetic acid/water (5:1:5, by vol.) and destained by repeated changes of a solution of acetic acid (7%, v/v) and methanol (5%, v/v).

Radioactivity measurements

Subcellular and submitochondrial fractions. A sample (50–100 μl) of each subcellular fraction was precipitated by addition of 1 ml of 10% (w/v) trichloroacetic acid. The precipitate was centrifuged at maximum speed in a bench centrifuge for 10 min and then was successively washed with 1 ml of 95% (v/v) ethanol, acetone and diethyl ether.

Submitochondrial samples containing approx. 3 mg of protein were supplemented with bovine serum albumin (3 mg) and the volume was adjusted to 0.4 ml with water before addition of 0.1 ml of 50% (w/v) trichloroacetic acid. Each precipitate was centrifuged at maximum speed in a bench centrifuge for 10 min and then successively washed with 1 ml of 10% (w/v) trichloroacetic acid and 1 ml of diethyl ether.

Each precipitate was then incubated at 90°C for 15 min in 1 ml of 10% (w/v) trichloroacetic acid. Final pellets were dissolved in 0.5 ml of 90% (w/v) formic acid and the solutions were transferred to scintillation vials. Each tube was washed with 0.5 ml

of water and the washings were added to the scintillation vial. Scintillation fluid (9 ml) was then added to each vial.

Gel slices. Each polyacrylamide gel was cut transversely into either 1 mm or 2 mm strips which were placed in scintillation vials and solubilized by incubating with 0.5 ml of H_2O_2 (100 vol.) at 80°C for 12 h. Formic acid (0.5 ml; 50% v/v) and 10 ml of FisoFluor were then added to each vial.

All radioactivity measurements were carried out on duplicate samples. The results are expressed as the mean of the d.p.m. values obtained. Radioactivity was measured with a Packard Tri-Carb 3375 or Packard Tri-Carb 3255 liquid-scintillation spectrometer. Corrections for quenching were made by using the external standards ratio method.

Protein half-life determinations

Protein half-lives were calculated by using the following equation, derived in Wilde *et al.* (1980).

$$\ln \left(\frac{\text{test}}{\text{control}} \right) \text{ isotope ratio} = K_D t$$

where K_D is the degradation rate and is related to the half life of the protein by $K_D = t_{1/2} / \ln 2$, where t is the time period when protein degradation was studied.

Protein determinations

Protein at high concentration (5–100 mg/ml) was determined by the method of Gornall *et al.* (1949) and at lower concentrations by the method of Sedmak & Grossberg (1977).

Results

Subcellular and submitochondrial fractionations

Livers were fractionated by the method of de Duve *et al.* (1955) to yield nuclear, mitochondrial, lysosomal, microsomal and particle-free supernatant fractions. Marker enzyme distributions were as expected for the fractionations (results not shown). The yield of mitochondrial-fraction protein was approximately the same (30 mg/g of liver) for each fractionation; 50–300 mg of mitochondrial-fraction protein was used for the submitochondrial fractionation. The distribution profiles of the submitochondrial marker enzymes for adult-rat mitochondrial fractionations are shown in Fig. 1. Submitochondrial marker enzymes for the weanling- and neonatal-rat mitochondrial fractionations also gave distribution profiles similar to those given in Fig. 1. One exception was noted to this distribution of marker enzymes, namely that, in the neonatal-rat mitochondrial fractionation, the distribution of cytochrome oxidase indicated a slightly increased contamination of the outer membrane fractions by

Fig. 1. Distribution profiles for monoamine oxidase, adenylate kinase, succinate dehydrogenase and malate dehydrogenase in the submitochondrial fraction

The submitochondrial fractionation procedure is described in the Materials and methods section. (a)–(d) Distribution of specific activities relative to the homogenate specific activity; (e)–(h) distribution of total enzyme activity in each submitochondrial fraction expressed as a percentage of the total enzyme activity. OM, outer membrane fraction; IMS, intermembrane space fraction; IM, inner membrane fraction; M, matrix fraction. Results represent the mean values from three experiments. (a) and (e), monoamine oxidase; (b) and (f), adenylate kinase; (c) and (g), succinate dehydrogenase; (d) and (h), malate dehydrogenase.

the inner membrane fraction. Table 1 shows the average protein distribution for the submitochondrial fractions derived from the rat liver mitochondrial fraction at the three post-natal ages.

Protein degradation during post-natal development

Samples of subcellular and submitochondrial fractions from animals at the three stages of post-natal development were subjected to polyacrylamide-gel electrophoresis in the presence of SDS. Isotope ratios were measured for the gel slices as described in the Materials and methods section. The mean $\ln(\text{isotope ratio})$ was determined for both test and control samples from the $\ln(\text{isotope ratio})$ for each gel slice. For the weanling age group two control experiments were performed (i.e. at 25 and 29 days *post partum*). The isotope ratios were very similar, and therefore a mean value from these two

Table 1. *Average protein distribution for the submitochondrial fractions derived from adult, weanling and neonatal rat liver*

Livers taken from adult (240g), weanling (25-day-old) and neonatal (8-day-old) rats were fractionated and the mitochondrial protein was subfractionated as described in the Materials and methods section. The protein content in each fraction is expressed as a percentage of the total mitochondrial protein recovered in the four submitochondrial fractions. Each individual protein estimation is the mean of triplicate determinations. Values given are the mean $\pm$ S.E.M. for the number of separate liver fractionations denoted in parentheses.

Fraction	Animal ...	Percentage of total mitochondrial protein		
		Adult	Weanling	Neonatal
Outer membrane		25.1 $\pm$ 2.1 (4)	12.5 $\pm$ 1.6 (6)	39.3 $\pm$ 4.3 (4)
Intermembrane space		15.2 $\pm$ 1.4 (4)	10.2 $\pm$ 1.8 (6)	9.2 $\pm$ 1.0 (4)
Inner membrane		46.0 $\pm$ 2.2 (4)	65.1 $\pm$ 3.2 (6)	33.4 $\pm$ 3.5 (4)
Matrix		14.2 $\pm$ 2.7 (4)	12.7 $\pm$ 1.3 (6)	18.1 $\pm$ 2.1 (4)

Table 2. *Mean half-lives of proteins from subcellular and submitochondrial fractions from adult, weanling and neonatal rat livers*

Details of the injection schedules and fractionation procedures for these developmental studies are given in the Materials and methods section. Mean half-lives of proteins in each fraction are calculated as described in Wilde *et al.* (1980) for test/control mean $\ln$ (isotope ratios) for protein subunits resolved by 10% (w/v) polyacrylamide-gel electrophoresis in the presence of SDS. Abbreviation: n.d., not determined.

Fraction or protein	Animals ...	$t_{1/2}$ (days)		
		Adult	Weanling	Neonate
Subcellular fractions				
Homogenate		2.5	2.9	4.2
Nuclear		2.4	7.3	3.7
Mitochondrial		4.8	5.5	>8.0
Microsomal		1.4	3.3	3.1
Lysosomal		3.1	4.2	4.0
Particle-free supernatant		>8.0	1.1	1.8
Mitochondrial fractions				
Outer membrane		2.6	2.6	>8.0
Intermembrane space		1.2	4.1	1.8
Inner membrane		6.3	4.4	3.6
Matrix		>8.0	3.9	3.7
Enzymes				
Monoamine oxidase subunit		2.8	2.7	>8.0
Pyruvate dehydrogenase				
α -Pyruvate decarboxylase subunit		4.5	>8.0	n.d.
β -Pyruvate decarboxylase subunit		5.0	>8.0	n.d.
Dihydrolipoyl acetyltransferase subunit		5.6	>8.0	n.d.
Dihydrolipoyl dehydrogenase subunit		5.0	>8.0	n.d.
Cytochrome oxidase subunits 1-6		>8.0	>8.0	n.d.

experiments was used in all calculations requiring control mean $\ln$ (isotope ratios).

Several factors, including the relative proportions of the two radiolabelled precursors administered to the animals and the ratio of incorporation of endogenous to exogenous arginine into protein, may influence the control mean $\ln$ (isotope ratio). These factors will also influence the test mean $\ln$ (isotope ratio). Since some of these factors may vary with the age of the animals [for example, the ratio of

incorporation of endogenous to exogenous arginine varies from 1:3 in the neonate to 1:1 in the adult rat (Drotman & Campbell, 1972)], the results are expressed as the ratio of the test mean $\ln$ (isotope ratio) to the control mean $\ln$ (isotope ratio) [test/control mean $\ln$ (isotope ratio)]. This mode of expression of the results allows the calculation of half-lives for enzyme subunits or protein mixtures as described in the Materials and methods section.

Half-lives for proteins from subcellular fractions

at three stages of post-natal development are shown in Table 2. It is clear that, in the adult-rat microsomal fraction, proteins are degraded more rapidly than are proteins in other subcellular fractions. The low rate of degradation of adult liver mitochondrial proteins has also been found by other workers (Arias *et al.*, 1969; Aschenbrenner *et al.*, 1970; Burgess *et al.*, 1978). A low rate of degradation was observed for the adult-rat particle-free supernatant proteins, which is at variance with the results of Arias *et al.* (1969) and Burgess *et al.* (1978). No satisfactory explanation was found for this apparently anomalous result. Homogenate, nuclear, mitochondrial, microsomal and lysosomal fractions exhibit decreased rates of protein degradation during post-natal development relative to the adult. Mean half-lives for the neonatal-rat subcellular proteins are in general longer than the respective weanling-rat half-lives. Neonatal-rat mitochondrial proteins have extremely long mean half-lives (greater than 8.0 days). Half-lives for submitochondrial protein fractions and the subunits of monoamine oxidase, pyruvate dehydrogenase and cytochrome oxidase are also shown in Table 2. Outer membrane and intermembrane space proteins have decreased degradation rates during post-natal development. Very little protein degradation could

be detected over the 4 day interval of the experiment in the adult-rat inner membrane and matrix fractions. Half-lives for the subunits of monoamine oxidase, pyruvate dehydrogenase and cytochrome oxidase in the adult-rat liver mitochondrial fraction are similar to the mean half-lives for proteins from their respective submitochondrial compartments. Monoamine oxidase has a half-life which approximates to the half-life for the mitochondrial outer membrane proteins at the three developmental stages. The rate of degradation of monoamine oxidase is therefore decreased in the neonate as is the rate of degradation of the outer membrane protein mixture. Pyruvate dehydrogenase subunits from weanling-rat liver have longer half-lives than the mean half-lives of proteins from the inner membrane fraction from which the enzyme complex was immunisolated. All enzyme subunits have very similar half-lives in adult and weanling animals. Little degradation of cytochrome oxidase subunits in adult or weanling rats could be measured (test/control isotope ratio range 0.8–1.16) over the 4 day experimental period.

Protein degradation in adult liver

Protein half-lives were also determined for liver subcellular and submitochondrial fractions by the

Table 3. *Mean half-lives of proteins from subcellular and submitochondrial fractions from adult rat livers*

Mean half-lives of proteins in each fraction are calculated as described in Wilde *et al.* (1980) for test/control mean $\ln(\text{isotope ratios})$ of trichloroacetic acid precipitates (a) and of protein subunits resolved by 10% (w/v) polyacrylamide gel electrophoresis in the presence of SDS (b). Details of injection schedules, fractionation procedures and extraction procedures for this adult study are given in the Materials and methods section. Half-lives (t_1) were calculated from the mean $\ln(\text{test/control isotope ratios})$ from two experiments. In each experiment four individual measurements of the isotope ratio for each trichloroacetic acid precipitate were made. Incorporation of radioactivity was in the range 200–5000 d.p.m./mg of protein for ^{14}C and $(30\text{--}100) \times 10^3$ d.p.m./mg of protein for ^3H . Abbreviation: n.d., not determined.

Fraction or protein	t_1 (days)	
	(a)	(b)
Subcellular fractions		
Nuclear	3.5	n.d.
Mitochondrial	3.5	4.1
Lysosomal	2.5	n.d.
Microsomal	2.2	n.d.
Particle-free supernatant	4.1	n.d.
Submitochondrial fractions		
Outer membrane fraction	2.1	2.9
Intermembrane space fraction	2.3	>8.0
Inner membrane fraction	6.3	8.0
Matrix fraction	>8.0	>8.0
Enzymes		
Monoamine oxidase subunit	—	2.3
Pyruvate dehydrogenase		
α -Pyruvate decarboxylase subunit	—	>8.0
β -Pyruvate decarboxylase subunit	—	7.4
Dihydrolipoyl acetyltransferase subunit	—	6.9
Dihydrolipoyl dehydrogenase subunit	—	>8.0

double-isotope procedure in adult steady state conditions (see the Materials and methods section).

Half-lives are shown (Table 3) for trichloroacetic acid-precipitated material and protein mixtures resolved by polyacrylamide-gel electrophoresis in the presence of SDS from subcellular and submitochondrial fractions. The trichloroacetic acid-precipitated material from the subcellular fractions was extracted with all solvents whereas the material from the submitochondrial fractions was less extensively treated (see the Materials and methods section). Clearly, turnover of proteins in the mitochondrial outer membrane is faster than that in the inner membrane fraction or in the solubilized matrix-protein fraction. There is a marked discrepancy in the mean half-lives of proteins in the intermembrane space fraction when determined by the two different methods. Only the value given by the trichloroacetic acid-precipitated material agrees with the half-life as measured by the non-steady-state modification of the double-isotope technique (Table 2).

Heterogeneity of protein degradation rates

Heterogeneity of protein degradation rates within each electrophoretically resolved protein mixture was estimated by comparison of the variance (F -test; $P < 0.05$) of the logarithmically transformed mean test and control isotope ratios. All adult subcellular fractions have heterogeneous rates of protein degradation. Heterogeneity of protein degradation rates in outer membrane and intermembrane space submitochondrial fractions is observed at all three ages studied. Little or no heterogeneity of protein degradation rates was measured in the inner membrane or matrix fractions at any stage of development. These fractions (the mitoplast) comprise over 50% of mitochondrial protein at each stage of development (Table 1).

Relationship of subunit size and degradation rate

No apparent correlation of subunit size with degradation rate was consistently observed for any subcellular or submitochondrial fraction (results not shown).

Discussion

The sensitivity of the double-isotope technique is dependent on the time interval between the administration of precursors, the time period after administration of the second precursor (Zak *et al.*, 1979), the choice of non-reutilizable precursors (Burgess *et al.*, 1978) and the absolute incorporation of the radioactive precursors into protein (Burgess *et al.*, 1978; Zak *et al.*, 1979). The choice of labelling periods for both radioactive precursors depends on the half-lives of the proteins of interest. Clearly no

pair of labelling intervals will give a satisfactory estimate of protein half-lives varying from a few hours to many days. Furthermore, as the protein half-life is proportional to the $\ln(\text{test/control isotope ratio})$ the accuracy of measurement of half-lives decreases rapidly as test/control isotope ratios approach a value of one or infinity.

The half-lives of liver mitochondrial proteins vary between 1 and 6 days (Mayer *et al.*, 1980) with average half-lives of liver mitochondrial proteins between 4 to 6 days (Swick *et al.*, 1968). The choice of both labelling periods in the experiments reported here was therefore a compromise between the need to achieve high absolute incorporation, particularly of ^{14}C , and the retention of adequate sensitivity indices (Zak *et al.*, 1979) particularly over the range of half-lives of mitochondrial proteins. The choice of interval after administration of the second label (^3H) means that the loss of this label from very rapidly degraded or secreted proteins will not be measured.

$\text{H}^{14}\text{CO}_3^-$ is a minimally reutilized precursor (Swick, 1958; McFarlane, 1963; Swick & Ip, 1974) which therefore is extremely useful, if not essential, for use as the first partner in a double-isotope couple to estimate relative protein turnover rates (Burgess *et al.*, 1978; Walker *et al.*, 1978). $\text{H}^{14}\text{CO}_3^-$ is rapidly incorporated into arginine and the decrease in specific radio-activity of this amino acid parallels the loss of protein radioactivity (Swick & Ip, 1974). Other amino acids are labelled by $\text{H}^{14}\text{CO}_3^-$, most notably glutamate/glutamine and in the mitochondrial proteins aspartate/asparagine, but the specific radioactivity of these amino acids is low relative to that of arginine (Swick & Handa, 1956). Despite the use of 12.5 mCi of $\text{H}^{14}\text{CO}_3^-$ per rat in these experiments the ^{14}C specific radioactivities for subcellular and submitochondrial proteins (Table 3) are similar to the specific radioactivities obtained by Burgess *et al.* (1978) using rats of similar size and only 5 mCi of $\text{H}^{14}\text{CO}_3^-$ per rat. The large loss of ^{14}C label (approx. 95%) in expired CO_2 during the first 4 h after injection (Gould *et al.*, 1949) may partially explain the similar specific radioactivities.

The method of Dice *et al.* (1978), used in the developmental study, requires that proteins are labelled in two animals when protein synthetic rates in a given tissue are identical. Furthermore, all the protein from a given tissue from each animal must be used for the preparation of the mixture before subsequent analysis. The second criterion is necessary during rapid growth, since the decline in the specific radioactivity of protein is a function of the rate of protein degradation and protein synthesis, whereas the decline in total radioactivity is a function of the rate of degradation only, provided that there is no isotope reutilization. These provisos were satisfied in the experiments reported here. The results (Table 2) with the non-steady state

modification of the method (Dice *et al.*, 1978) give essentially the same degradation rates in adult tissue fractions as does the steady state method (Table 3; Burgess *et al.*, 1978; Walker *et al.*, 1978). Variation between the half-lives determined for adult-rat subcellular and submitochondrial protein fractions (Tables 2 and 3; Burgess *et al.*, 1978) may be due to the different methods of half-life estimation, using either trichloroacetic acid precipitates or protein subunits resolved by 10% (w/v) polyacrylamide-gel electrophoresis. Half-lives determined for the acid precipitates (Table 3; Burgess *et al.*, 1978) may be biased by more hydrophilic proteins due to selective loss of hydrophobic proteins during the extensive washing of the precipitates with organic solvents (see the Materials and methods section) to remove lipids. The $t_{\frac{1}{2}}$ determined for monoamine oxidase in adult rat liver mitochondrial fractions by the steady state method (2.3 days; Table 3), by the non-steady state method (2.8 days; Table 3), and by the pargyline irreversible inhibition method (2.4–2.6 days; Tipton & Dela Corte, 1979) shows the reproducibility and accuracy of the determination of protein degradation that can be achieved by the different procedures for single proteins. A further complication of the experiments is that fractionation of liver must obviously precede mixing when the rates of degradation of subcellular and submitochondrial proteins are compared. In each case two sets of liver proteins were mixed after fractionation. Detailed electrophoretic analysis of many subcellular protein fractions limits the number of animals that may be studied. The post-natal development study must therefore be considered with this in mind.

The proteins in the microsomal fraction are the most rapidly degraded subcellular-fraction proteins (Tables 2 and 3). The proteins of the mitochondrial outer membrane have much higher average rates of protein degradation than the mitochondrial inner membrane and matrix proteins (Tables 2 and 3). The higher average rate of protein degradation in the mitochondrial outer membrane relative to the inner membrane is in agreement with measurements for proteins derived from the outer and inner membranes by several workers (Brunner & Neupert, 1968; de Bernard *et al.*, 1969; Druyan *et al.*, 1969).

The average decrease in the mean rate of degradation of subcellular and submitochondrial proteins (with the exception of particle-free supernatant proteins and matrix proteins) is 1.60 ± 0.34 ($n = 7$) -fold for liver proteins from weanling rats and 2.34 ± 0.63 ($n = 6$) -fold for liver proteins from neonatal rats. These findings are in general agreement with the results of Conde & Scornik (1977) for liver proteins in neonatal mice and of Scornik & Botbol (1976) and Tauber & Reutter (1978) on protein degradation in regenerating mouse and rat liver respectively. The decrease in the rate of protein

degradation is similar in different weanling-rat subcellular fractions and in several neonatal-rat subcellular fractions, implying a degree of co-ordinated change in the rate of degradation for proteins of different subcellular fractions. The half-life for neonatal-rat mitochondrial proteins of more than 8.0 days is comparable with that of 15 days determined for mitochondrial proteins in rapidly growing adrenal cortex (Canick & Villee, 1974). The low rate of protein degradation for neonatal-rat mitochondrial fraction proteins may be a consequence of a decrease in some rate-limiting step within a cycle of mitochondrial maturation and division (Pollak, 1976). There is some evidence for a high rate of mitochondrial protein degradation in foetal rat liver (Pollak, 1976). The observed decrease in mitochondrial protein degradation in the neonate and weanling (Table 3) and the subsequent increase in the adult gives an overall developmental profile of protein degradation in mitochondrial fraction proteins similar to that observed for fatty acid synthase and some cytosol proteins in terminally differentiating mammary gland explants in organ culture (Speake *et al.*, 1975, 1976; Paskin & Mayer, 1978; Wilde *et al.*, 1980).

The results in Table 2 and 3 concerning the half-lives of pyruvate dehydrogenase subunits indicate co-ordinate degradation of this multi-subunit enzyme complex. The rate of degradation of both monoamine oxidase and pyruvate dehydrogenase subunits is also decreased during post-natal development. Virtually no degradation of cytochrome oxidase in either adult or weanling rats can be measured with the 4-day interval between precursor administration used in these experiments.

Measurements of the degree of heterogeneity of protein degradation rates within a subcellular or submitochondrial fraction are complicated by two factors of opposite bias. Since each subcellular or submitochondrial fraction has a different mean rate of protein degradation (Tables 2 and 3) a certain amount of heterogeneity of degradation rates within a subcellular fraction can be expected from cross-contamination of subcellular and submitochondrial fractions. It is not possible in these experiments to ascertain accurately the contribution of this cross-contamination to the heterogeneity of protein degradation rates. Another factor that affects measurements of heterogeneity of protein degradation rates is the problem of averaging isotope ratios when separating polypeptides by electrophoresis on the basis of subunit size (Roberts & Yuan, 1974; Horst & Roberts, 1979). Between submitochondrial compartments, where the problems of protein contamination and averaging are decreased, there is heterogeneity of protein degradation rates in the mitochondrial outer membrane and intermembrane space fraction, and

little or no heterogeneity of protein degradation rates in the inner membrane and matrix fractions (results not shown) in agreement with previous work (Druyan *et al.*, 1969; Gross *et al.*, 1969; Aschenbrenner *et al.*, 1970).

The real degree of heterogeneity of protein degradation rates can only be estimated by better analytical resolution (i.e. two-dimensional electrophoresis) and use of different intervals between isotope administration in the double isotope procedure.

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