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Haem *a*, Cytochrome *c* and Total Protein Turnover in Mitochondria from Rat Heart and Liver

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Haem *a* and cytochrome *c* were isotopically labelled in mitochondria from rat heart and liver after injection of δ-amino[2,3-$^3H_2$]laevulate, a specific haem precursor. [*guanido*-^{14}C]Arginine or L-[4,5-$^3H_2$]leucine were used to label mitochondrial proteins. Half-lives were measured from biological decay *in vivo* and were similar (5.5–6.2 days) for haem *a*, cytochrome *c* and [^{14}C]arginine-labelled proteins. Labelling of hepatic mitochondrial proteins with [$^3H_2$]leucine resulted in a prolonged apparent half-life.

The hypothesis that the mitochondrion turns over as a unit was first advanced by Fletcher & Sanadi (1961) and was based on their observations that several components of rat liver mitochondria showed similar exponential decay rates. The calculated half-lives of total phospholipids, soluble and insoluble mitochondrial proteins, and cytochrome *c* labelled with [^{35}S]methionine or [^{14}C]acetate were all about 10 days. These studies used radioactive precursors that now are known to be efficiently reutilized for resynthesis of macromolecules, and hence reutilization may have considerably prolonged the observed turnover rates. We have reported a modified approach, based on measuring turnover rates of specific mitochondrial cytochromes labelled with δ-amino[3,5-$^3H_2$]laevulate (Druyan, DeBernard & Rabinowitz, 1969). δ-Aminolaevulate is a specific haem precursor, efficiently incorporated into the prosthetic group of cytochromes. Moreover, haem is degraded to bilirubin, and the radioactive label is not reutilized. We have previously shown that the half-lives of cytochromes *b* and *c*, both localized on the inner mitochondrial membrane, were similar, about 5–6 days in rat liver (Druyan *et al.* 1969). In contrast, cytochrome b_5, found on the outer mitochondrial membrane, turned over more rapidly ($t_{\frac{1}{2}}$ 4.4 days). In this paper we extend these findings to haem *a* in rat liver mitochondria, and haem *a* and cytochrome *c* in heart mitochondria. The turnover rates of these cytochromes are compared with the turnover rates of total mitochondrial protein labelled with two different amino acid precursors: L-[4,5-$^3H_2$]leucine and [*guanido*-^{14}C]arginine. Since [*guanido*-^{14}C]-arginine is a non-reutilizable protein precursor (Schimke, 1964), differences in measured turnover rates with these two precursors yield an index of [$^3H_2$]leucine reutilization after protein degradation in liver and heart.

MATERIALS AND METHODS

Liver mitochondria were isolated from adult, female Sprague–Dawley rats by using the method of Parsons & Williams (1967) as previously described (Druyan *et al.* 1969). Microsomal contamination, with glucose 6-phosphatase activity as a marker, was less than 5% with this technique (Druyan *et al.* 1969). A 10% (w/v) heart muscle homogenate was prepared in 0.27M-sucrose, 10mM-tris chloride buffer, pH 7.5, 10mM-KCl, 0.1mM-EDTA. The homogenate was incubated with Nagarse (Enzyme Development Corp., New York, N.Y., U.S.A.) (0.5mg/ml) at 0°C for 20min (Chance & Hagihara, 1963). The pH was re-adjusted to 7.5 with NaOH, and after re-homogenization cell debris was removed by centrifugation at 800*g*. This step was repeated. Mitochondrial pellets were obtained by centrifugation at 9000*g*; pellets were twice resuspended and recentrifuged. Two more centrifugations were made at 800*g*, and the pellets were discarded. Mitochondria were collected after a final centrifugation at 9000*g*. All centrifugation steps were made for 10min at 4°C. Mitochondrial purity was checked by electron microscopy; contaminating cellular structures were not seen.

Cytochrome *c* was extracted from mitochondria with 0.15M-KCl, and the protein was purified by chromatography on XR-64 resin (Paul, 1955). Eluted cytochrome *c* was dialysed overnight against 10mM-sodium phosphate buffer, pH 7.5, before analysis. Cytochrome *c* concentration was determined from its extinction after reduction with dithionite: $(E_{550}-E_{535}) = 20.4\,mM^{-1}$ (Margoliash & Frohwirt, 1959). With this technique both protein concentration and radioactivity reflect measurements made

of the haem group rather than the holoprotein. Incorporation of radioactive aminolaevulate appears specific for the prosthetic group of haemoproteins (Lester, Daly, Little & Troxler, 1967; Levin & Kuntzman, 1969; Druyan *et al.* 1969) and hence, separation of non-haemoproteins is not critical to the measurement of cytochrome *c* specific radioactivity with the methods employed in this study. Contamination of cytochrome *c* samples by other haemoproteins was not detected; absorption spectra between 400 and 600nm were recorded for each preparation of cytochrome *c* and showed the characteristic spectra for this protein. After extraction of cytochrome *c*, the residual pellets were homogenized with acetone–water (4:1, v/v) for 10min and centrifuged for 10min at 6500*g*. The pellets were dried under a stream of N_2 and were further freed from lipid by three extractions with chloroform. Haem was extracted twice from lipid-depleted mitochondria with chloroform–pyridine (2:1, v/v) for 30min each time (York, McCoy, Taylor & Caughey, 1967). After this step, light was excluded from vessels that contained haem. Haem was concentrated by evaporation *in vacuo* at 30°C; the dry residue was dissolved in several drops of chloroform–pyridine (1:1, v/v). The haem solution was applied to a column (0.6cm × 6cm) containing Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with chloroform–pyridine. Haem *a* was eluted with the lower phase of the following mixture: 50ml of pyridine, 25ml of chloroform, 25ml of 0.1% $NaHCO_3$, 3.3ml of 2,2,4-trimethylpentane (York *et al.* 1967). Haem *a*, which appeared as a green band, was eluted before and distinct from haem *b*, a red–brown band. Fractions of haem *a* were pooled, washed with 0.05M-acetic acid, and were evaporated to dryness *in vacuo*. The haem *a* was dissolved in pyridine and reduced with dithionite. Absorption spectra were recorded with a Cary model 14 spectrophotometer, and the concentration of haem *a* was calculated from the following relationship:

$$E_{583} - \frac{E_{550}+E_{510}}{2} = 21.7\,\text{mM}^{-1} \text{ (York } et\ al. \text{ 1967).}$$

Separate experiments were performed with [$^3H_2$]leucine and [*guanido*-^{14}C]arginine to label mitochondrial proteins. Mitochondrial protein was precipitated with trichloroacetic acid and freed of lipid by solvent extractions (Zak, Grove & Rabinowitz, 1969). A sample of protein was dissolved in formic acid and measured for radioactivity. The protein concentration of a sample of the same solution was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as a reference standard.

Rats were given intraperitoneal injections of labelled precursors. For labelling haem *a* and cytochrome *c*, each rat received 100μCi of δ-amino[2,3-$^3H_2$]laevulate (Schwarz Bioresearch Inc., Mount Vernon, N.Y., U.S.A.); for measurement of total mitochondrial protein turnover, each rat received 10μCi of [*guanido*-^{14}C]arginine (Amersham Searle, DesPlaines, Ill., U.S.A.) or 50μCi of [$^3H_2$]-leucine (Schwarz Bioresearch Inc.). Rats were killed at 48h intervals, after having been starved overnight. The radioactivity of ^{3}H-labelled haem *a*, cytochrome *c* and total mitochondrial proteins (from [$^3H_2$]leucine) was measured with a Packard 544 liquid-scintillation system. Results were corrected for quenching, and are expressed as d.p.m. The radioactivity of [*guanido*-^{14}C]arginine-labelled proteins was measured with a low-background gas-flow system (Beckman Low Beta II). Less than 2mg of protein was dried on each planchet; under these conditions, there is no self-absorption.

RESULTS

In preliminary experiments, haem *a* was purified by solvent extraction (Rawlinson, 1949) or by chromatography on celite (York *et al.* 1967) or silicic acid (Connelly, Morrison & Stotz, 1958). With small scale preparations, celite chromatography after pyridine–chloroform extraction gave haem *a*, but more consistent results were obtained when Sephadex LH-20 was substituted for celite. Dithionite-reduced haem *a* samples showed absorption maxima at 430 and 583nm ($E_{430}/E_{583} \simeq 5$).

Turnover rates for haem *a* and cytochrome *c* were measured with mitochondria isolated from both rat heart and liver. For liver, calculated half-lives were 5.8 days for haem *a* and 5.6 days for cytochrome *c*, as shown in Fig. 1. These values are not significantly different, $P > 0.5$. [P values were determined as described by Goldstein (1964).] With heart mitochondria, turnover rates of haem *a* and cytochrome *c* were very similar, as shown in Fig. 2. Haem *a* was renewed with a 5.9 day half-life; the half-life of cytochrome *c* was 5.8 days. Turnover rates for heart muscle haem *a* and cytochrome *c* are not significantly different, $P > 0.5$.

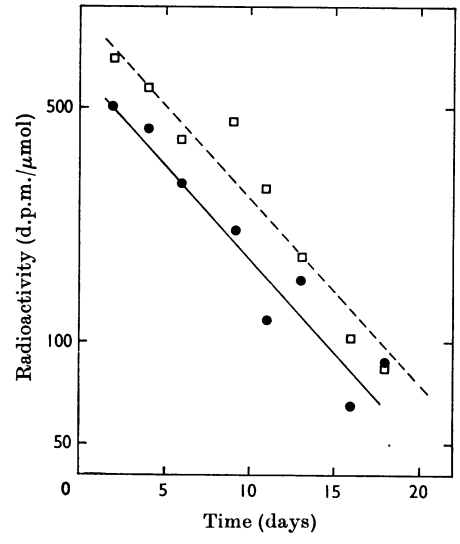

Fig. 1. Haem *a* (●) and cytochrome *c* (□) turnover in liver mitochondria after labelling with δ-amino[3,5-$^3H_2$]-laevulate. Half-lives were 5.6 days for haem *a* and 5.5 days for cytochrome *c*.

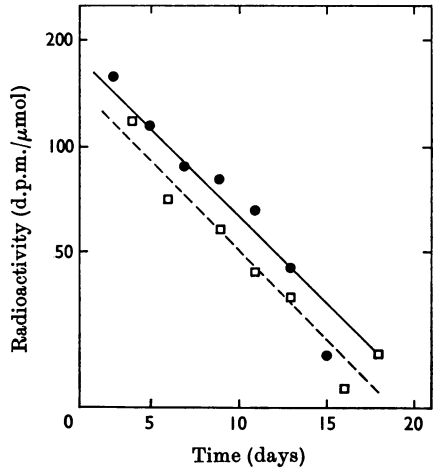

Fig. 2. Turnover of δ-amino[3,5-$^3H_2$]laevulate-labelled haem *a* (●) and cytochrome *c* (□) in heart mitochondria. For haem *a*, half-life was 5.9 days; for cytochrome *c*, half-life was 5.8 days.

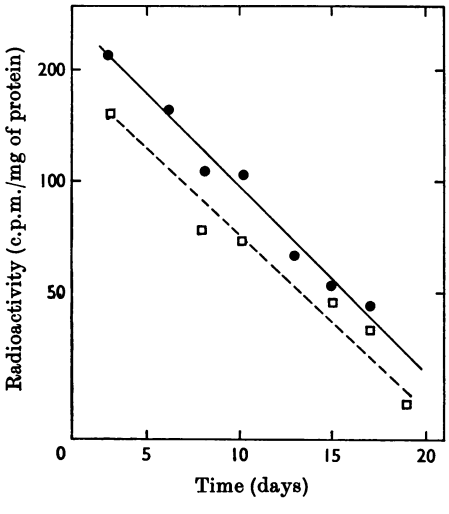

Fig. 3. Total protein turnover in heart mitochondria. After [$^3H_2$]leucine incorporation (●), half-life was 6.1 days; after [*guanido*-^{14}C]arginine incorporation (□), half-life was 6.2 days.

To measure turnover of total mitochondrial proteins, separate experiments were performed, and radioactivity incorporated into protein was counted after injecting [$^3H_2$]leucine or [*guanido*-^{14}C]arginine. In experiments with heart mitochondria, similar protein half-lives were measured with [$^3H_2$]leucine or [*guanido*-^{14}C]arginine, as shown in Fig. 3. With liver mitochondria, the apparent turnover rate of mitochondrial protein

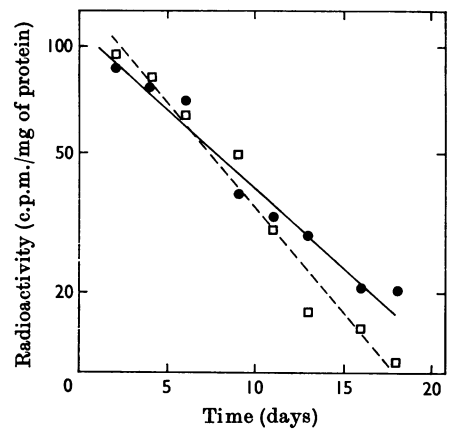

Fig. 4. Total protein turnover in liver mitochondria. After [$^3H_2$]leucine incorporation (●), half-life was 7.0 days; after [*guanido*-^{14}C]arginine incorporation (□), half-life was 5.0 days.

differed, depending upon which radioactive precursor was injected (Fig. 4). With [$^3H_2$]leucine the half-life was 7.0 days, and with [*guanido*-^{14}C]-arginine the half-life was 5.0 days, significently less ($P<0.05$).

DISCUSSION

In this paper we have shown that haem *a* and cytochrome *c* of rat liver and heart turn over at essentially the same rates. These data, added to our previous observations that rat liver haem *b* and cytochrome *c* also turn over with identical half-lives of about 5–6 days, provide further support for our hypothesis that the inner mitochondrial membrane, or a significant fraction thereof, is synthesized and degraded as a unit (Gross, Getz & Rabinowitz, 1969). It is also significant that the turnover rates of total mitochondrial proteins in liver and in heart, which in turn reflect predominantly the renewal of the inner mitochondrial membrane, are also of the same magnitude. Further, rat heart mitochondrial DNA is renewed at a similar rate, with a half-life of 6.7 days. Thus, in heart mitochondria, δ-amino-[^{3}H]laevulate-labelled cytochromes *a*, *b* and *c*, total mitochondrial protein and mitochondrial DNA all turn over at similar rates. The same appears to be true for liver mitochondria, but not for mitochondrial DNA (Gross *et al.* 1969). The longer half-life of liver mitochondrial DNA (9.4 days) (Gross *et al.* 1969) may reflect reutilization of precursor [^{3}H]thymidine in this organ.

It is, however, apparent that matrix and outer mitochondrial membrane components turn over independently of the inner mitochondrial membrane. We have shown that cytochrome b_5,

localized in the outer membrane, is renewed with a half-life of 4.4 days, which is significantly faster than the turnover of haem *b* and cytochrome *c*.

These observations agree with measurements of total protein turnover of inner and outer membranes made after labelling with the reutilized precursors [^{35}S]methionine (Brunner & Neupert, 1968) or [^{14}C]leucine (Beattie, 1969), and with experiments (DeBernard, Getz & Rabinowitz, 1969) that used a double-label technique (Schimke, Ganschow, Doyle & Arias, 1968) from which conclusions independent of reutilization may be made.

δ-Aminolaevulate synthetase, an enzyme found in the matrix fraction (McKay, Druyan, Getz & Rabinowitz, 1969) turns over with a half-life of only 70min (Tschudy, Marver & Collins, 1965). This enzyme, and probably other matrix enzymes, can therefore be degraded independently of the mitochondrial membrane systems.

Our data also indicate that reutilization of amino acid precursors for protein synthesis differs in heart and liver. Measurement of turnover of total mitochondrial proteins in liver and heart by using [$^{3}H_2$]leucine and [*guanido*-^{14}C]arginine clearly shows differences in patterns of reutilization of precursor amino acids in these two organs. Turnover measurements may be artificially prolonged by significant isotope reutilization after protein degradation. Labelling of liver mitochondria with the non-reutilized precursor, [*guanido*-^{14}C]arginine, yielded more rapid turnover rates than labelling with [$^{3}H_2$]leucine. These results resemble data reported by Swick, Rexroth & Stange (1968) and Arias, Doyle & Schimke (1969), that showed a more rapid protein turnover rate with [*guanido*-^{14}C]arginine in liver. In contrast, protein turnover rates in heart mitochondria are identical after [$^{3}H_2$]leucine or [*guanido*-^{14}C]arginine labelling. Based on these data, we now suggest that heart mitochondria do not readily reutilize some amino acids, nor perhaps nucleic acid precursors.

During preparation of this manuscript Kadenbach (1969) reported a 43-day half-life for heart cytochrome *c* after [^{14}C]lysine incorporation. Protein turnover in rat heart mitochondria has been measured (Niklas, Quincke, Maurer & Neyen, 1958) and results similar to ours have been reported. The fivefold disparity between half-lives of heart cytochrome *c* is not presently explicable. Possibilities include an extraordinary reutilization of [^{14}C]lysine, or differing turnover rates for the haem and apoprotein moieties of cytochrome *c*. Preliminary experiments (R. Druyan, unpublished work) suggest that the latter possibility is unlikely. Incorporation of radioactive labels into the haem and protein moieties of cytochrome *c* occurs at similar rates, and both rates are similarly decreased by inhibition of protein synthesis.

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