Synthesis and Degradation of Mitochondrial Components in Hypertrophied Rat Heart

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The accumulation of inner mitochondrial components of rat heart was studied 1 and 3 days after constriction of the ascending aorta of rats. By 1 day after aortic constriction, the activities of three mitochondrial respiratory enzymes/mg of cardiac homogenate protein were increased; after 3 days, specific activities had levelled off or decreased. Selective accumulation of inner mitochondrial membrane components 24h after aortic constriction was further indicated by increased left ventricular cytochrome *c* concentration (nmol/mg of protein). By 3 days after surgery, cytochrome *c* concentration was significantly diminished. Low-temperature spectroscopy of isolated mitochondria showed that the ratios of cytochromes *c*, *b* and *a*+*a*₃ remained unchanged after aortic constriction, suggesting that cytochrome *c* was a good indicator of the response of the other mitochondrial inner-membrane cytochromes as well. The effect of cardiac hypertrophy on the turnover of cytochrome *c* was also examined. Cytochrome *c* was labelled in its haem group with δ -amino[2,3-³H₂]laevulinate 3 days before aortic constriction. By 1 day after surgery the total ventricular radioactivity in cytochrome *c* of aortic banded animals was significantly higher than in sham-operated controls, indicating a decreased degradation rate in the former during the first postoperative day. δ -Aminolaevulinate was shown to be a particularly suitable precursor for such turnover studies, since it results in rapid pulse-labelling of cytochrome *c* (peak activity in 90min), is rapidly removed from the precursor pool ($t_{\frac{1}{2}}$ = 30min) and is not reutilized.

A substantial increment in left-ventricular mass may be produced rapidly by a variety of experimental procedures that impose a pressure or volume overload on the heart (Rabinowitz & Zak, 1972). Associated with the left-ventricular enlargement are increases in RNA polymerase activity (Nair *et al.*, 1968), in the rates of RNA (Fanburg & Posner, 1968; Koide & Rabinowitz, 1969) and protein synthesis and in the total RNA, DNA and protein content of the heart (Zak & Fischman, 1971; Morkin *et al.*, 1972).

The mechanisms by which RNA transcription and protein synthesis are stimulated are still unknown. It is apparent, however, that there is a good correlation between the increment in ventricular wall tension and the associated increment in the rate of oxidative metabolism (Coleman *et al.*, 1969), and cardiac muscle growth. It was therefore decided to evaluate the effects of rapid cardiac enlargement on the synthesis and degradation of cardiac mitochondria.

The present paper examines the question whether or not mitochondrial components accumulate in parallel with other cell components in the early period after induction of rapid cardiac enlargement by constriction of the ascending aorta. Relative changes in mitochondrial mass are determined from measurements of mitochondrial cytochrome content and respiratory enzyme activities.

We also tried to evaluate whether the accumulation of cytochrome *c* during hypertrophy is secondary to changes not only in the rate of its synthesis, but also of its destruction. Cytochrome *c* is probably a good indicator of the response of a significant portion of the inner mitochondrial membrane (Rabinowitz & Swift, 1970). Most investigations of turnover of muscle components are difficult to interpret because of the possibility of reutilization of the labelled precursor used in the measurements. To avoid this difficulty, we have examined cytochrome *c*, specifically labelled in its haem group with δ -amino[2,3-³H₂]laevulinate which is a non-reutilizable precursor.

Materials and Methods

Surgical procedure

Supravalvular aortic stenosis was produced in female Sprague-Dawley rats, weighing 200–220g, by the method described by Nair *et al.* (1968), with the following modifications: after the rats were anaesthetized, an intrapharyngeal tube was inserted through which an O₂+CO₂ (95:5) mixture was delivered at the rate of 3 litres/min. The lungs could be inflated by closing the mouth and nostrils. Gastrointestinal distension was prevented by adhesive tape placed around the abdomen. Rats were respired

in this manner for 1 min before the chest was opened. Surgical procedures were usually completed within 1 min and always in less than 3 min. Only animals that were resuscitated within 1 min after surgery were used for experiments.

Measurement of left-ventricular cytochrome content

Cytochrome *c* was quantitated as described by Aschenbrenner *et al.* (1971) by using about one-third of minced left ventricular tissue from two rats hearts. The remaining left ventricular tissue was used to isolate a partially purified mitochondrial preparation for low-temperature spectroscopy. Mitochondria were prepared as described by Aschenbrenner *et al.* (1971), except that the Nagarse (proteolytic enzyme) treatment was omitted and a single resuspension and centrifugation was performed. The preparations were free of haemoglobin, and spectral analysis was not significantly affected by the small contamination with other cellular-membrane fractions.

For low-temperature spectroscopic analyses, the mitochondrial pellets were resuspended in 1.0 ml of 0.02 M-sodium phosphate buffer, pH 7.4, containing 1% (w/v) sodium deoxycholate. After reduction with dithionite, 0.7 ml of mitochondrial suspension was placed into a plexiglass-aluminium cuvette (Claisse *et al.*, 1970) having a 4 mm light-path, and quickly plunged into liquid N₂. When freezing was complete, the cuvette was transferred to the sample compartment of a Cary model 14 recording spectrophotometer modified to accommodate an unsilvered Dewar flask containing liquid N₂. The 'absolute' spectrum was recorded from 630 to 500 nm, by using appropriate filters to balance the turbidity of the frozen sample. Relative amount of cytochromes *a*, *b* and *c* were determined by using the method described by Claisse *et al.* (1970), with the exception that 540 and 630 nm were used as base-line construction points.

Respiratory enzyme assays

Cytochrome oxidase activity was determined by a modification of the method outlined by Cooperstein & Lazarow (1951) and as described by Rabinowitz *et al.* (1969). Succinate cytochrome *c* reductase activity was determined by the method of Rabinowitz & DeBernard (1957). In some cases Lubrol, a non-ionic detergent (I.C.I. Organics Inc., Providence, R.I., U.S.A.), was added to activate cytochrome oxidase or succinate cytochrome *c* reductase activity (Schnaitman & Greenawalt, 1968) in the homogenate or mitochondria. It was found experimentally that 0.5 mg of Lubrol/mg of homogenate or mitochondrial protein provided maximal enzyme activation. Incubation with Lubrol was performed for 1 min at 37°C before the material was added to the assay mixture. Succinate dehydrogenase activity was determined by

the Pennington (1961) modification of the method developed by Nachlas *et al.* (1960).

Determination of radioactivity of free δ -amino laevulinate

We injected δ -amino[2,3-³H₂]laevulinate (specific radioactivity of 2.64 Ci/mol; New England Nuclear Corp., Boston, Mass., U.S.A.) in sterile saline into the tail veins of 200 g rats. Rats were killed, at time-intervals indicated in the Figures and Tables, by a blow on the head, the hearts were excised, immersed into ice-cold saline, blotted and weighed. Blood was removed by retrograde coronary-artery perfusion with ice-cold saline for 1 min by using a syringe inserted into the aorta. Hearts were then frozen and stored at -20°C until analysed. Hearts were thawed and then minced with scissors and ground with sea sand in a precooled mortar. Free δ -aminolaevulinate was extracted with 5 ml of ice-cold 0.5 M-HClO₃ for 30–60 min. The homogenate was centrifuged at 500 g for 5–10 min and the pH of the supernatant adjusted to 7.0 with 60% (w/v) KOH. The precipitate was left at 4°C for at least 1 h and then removed by centrifugation for 10 min at 500 g. The δ -amino[2,3-³H₂]laevulinate in the extract was then separated by the method of Mauzerall & Granick (1956), except for the following modifications: the entire HClO₃ extract was loaded on a column (1 cm × 2 cm) of Dowex 2; δ -aminolaevulinate was eluted from a column (1 cm × 2 cm) of Dowex 50 with 5 ml of 1 M-sodium acetate, and the volume was adjusted to 6 ml. For determination of radioactivity, 0.5 ml of eluate was counted in 15 ml of scintillant described by Bray (1960).

*Determination of specific radioactivity of cytochrome *c**

δ -Amino[2,3-³H₂]laevulinate (50 μ Ci/100 g body wt.) (New England Nuclear Corp., sp. radioactivity 2.6 Ci/mmol; or Schwarz, Bioresearch Inc., Mount Vernon, N.Y., U.S.A., 7.5 Ci/mmol) were administered either intraperitoneally or intravenously. The uniformity of the intravenous injection was checked by determining the radioactivity in blood samples (20 μ l) from the tail vein 10 min after injection. Protein was precipitated with 1 ml of 5% (w/v) trichloroacetic acid and 0.5 ml of the supernatant was counted in Bray's (1960) scintillant. Animals having markedly aberrant amounts of radioactivity in the blood were discarded.

At appropriate time-periods the rats were killed, and the hearts were processed and frozen as described above for measuring free δ -aminolaevulinate radioactivity. The hearts were thawed, finely minced and then homogenized with 20 strokes in a Potter-Elvehjem teflon-glass homogenizer in 10 ml of 0.15 M-KCl and 0.02 M-sodium phosphate buffer,

pH 7.0. The residue was removed by centrifugation at 500g for 10min and the pH of the supernatant was adjusted to 4.2–4.5 with 0.5M-H₂SO₄ and left overnight at room temperature. The precipitate was then removed by centrifugation at 65000g for 10min and the pH of the supernatant was adjusted to 7.0 with solid Na₂HPO₄. The solution was dialysed for 90min against 1 litre of water and for 90min against 0.02M-sodium phosphate buffer, pH 7.0. Dialysis solutions were changed every 30min. The solution was then centrifuged for 10min at 45000g and adsorbed on Amberlite XE-75 (NH₄⁺ form). The column (0.3cm×0.5cm) was washed four times with 10ml of water and four times with 10ml of 0.02M-NH₃. The cytochrome *c* was eluted with approx. 1 ml of 0.8M-NH₃ directly into dialysis tubing and dialysed for 1–2h against 0.02M-sodium phosphate buffer, pH 7.0, with two changes. After centrifugation for 10min at 45000g, a portion (0.2ml) was taken for potassium ferricyanate-oxidized–dithionite-reduced difference spectrum for measurement of cytochrome *c* content, as described by Aschenbrenner *et al.* (1971). A portion of the remaining solution (approx. 0.8ml) was counted for radioactivity in 10ml of Scintisol (Isolab Inc., Elkhardt, Ind., U.S.A.). All dialysis tubing used was pretreated by boiling in 0.1M-NH₄HCO₃ to decrease its permeability to cytochrome *c*.

For turnover experiments, tissue cytochrome *c* content and specific radioactivity were measured in the same sample. In this case, quantitative extraction of cytochrome *c* was obtained by using solutions containing 0.4M-NaCl and 1% (w/v) sodium deoxycholate, and by sonication of samples as described by Aschenbrenner *et al.* (1971). Cytochrome *c* was measured spectrophotometrically in the extract after precipitation of interfering proteins at pH 4.5. A portion of the extract was then purified on Amberlite XE-64 and the specific radioactivity measured as described above.

Determination of radioactivity

Radioactivity was assayed with a model 332 Packard Tri-Carb liquid-scintillation spectrometer. Corrections for quenching were made by use of an external standard. Radioactivity of ³H was measured at an efficiency of about 20%.

Results

Respiratory enzyme activity after aortic banding

Accurate measurement of respiratory enzyme activity in homogenates and mitochondria obtained from heart muscle is difficult because of problems in achieving standardized homogenization and consistent activation of latent enzyme activities. To try to evaluate these difficulties cytochrome oxidase and succinate cytochrome *c* reductase was measured in

homogenates and mitochondria prepared with and without the proteolytic enzyme Nagarse (Chance & Hagihara, 1961), and with the addition of the non-ionic detergent Lubrol (Schnaitman & Greenawalt, 1968). The concentration of Lubrol used (0.5mg/mg of protein) gave optimum (50–100%) stimulation of enzyme activity. Higher concentrations were inhibitory. The extent of stimulation was the same in preparations from control and hypertrophied hearts. The pattern of results obtained when Nagarse was used in the preparation was the same as that observed in its absence. Since considerable variations in absolute amounts of enzyme activity were observed from day to day, all results are expressed as a percentage of values of sham-operated control samples that were processed and measured in parallel with experimental samples.

Table 1 shows that the homogenate specific activities (units/mg of protein) of cytochrome oxidase, succinate cytochrome *c* reductase and succinate dehydrogenase increased 1 day after surgery. At 3 days, cytochrome oxidase and succinate dehydrogenase activities/mg of homogenate protein were still increased, whereas succinate cytochrome *c* reductase activity had declined below control values. When total cardiac enzyme activity is calculated by taking increasing heart weight into consideration, it is found that total activity increases to an even greater extent, and that the increase begins to level off on the third day.

No significant differences between hypertrophied and control hearts were observed in the specific activity of the three respiratory enzymes measured in purified mitochondria, either 1 or 3 days post-operatively (results not shown).

These results suggest that mitochondrial mass increased in early hypertrophy and that the increase was due to an increase in the number of functioning units rather than to the increased activity of pre-existing units. During the first day after banding of the aorta, mitochondrial mass appeared to increase to a greater extent than other myocardial proteins as determined by an increase in respiratory-enzyme activity/mg of myocardial protein. However, this conclusion must be considered tentative because of uncertainties in the enzyme assays. Alterations in the cell composition of hypertrophied hearts could lead to enhanced release of active respiratory particles during homogenization, or to greater activation of measured respiratory enzyme activity. Because of these uncertainties, we measured changes in total cardiac cytochrome content in hypertrophy.

Left-ventricular cytochrome content after aortic constriction

Left-ventricular cytochrome *c* content was measured spectrophotometrically after quantitative extraction from tissue homogenates. Estimates of total

Table 1. *Effect of aortic constriction on mitochondrial respiratory enzyme activities in homogenates of ventricular muscle*

Aortic banded and sham-operated control rats were killed 24 and 72 h after surgery and respiratory enzyme activities measured in ventricular homogenates as described in the Materials and Methods section. Mean enzyme activities for sham-operated controls were for cytochrome oxidase, $77.3 \text{ s}^{-1}/\text{mg}$ of protein; succinate cytochrome *c* reductase $0.256 \mu\text{mol}$ of cytochrome *c* reduced/min per mg of protein and for succinate dehydrogenase 23.6 mg of formazan produced/h per mg of protein. Values are given $\pm$ s.e. with the numbers of rats in parentheses. *P* values were obtained by use of the sign test: * $P < 0.05$; ** $P < 0.1$. Average percent hypertrophy at 24 and 72 h after operation was 18 and 35% respectively.

Enzyme	Time after operation (h) ...	Mean percentage difference from sham-operated controls	
		24	72
Cytochrome oxidase		$+24.7 \pm 2.6$ (9)*	$+19.8 \pm 1.9$ (7)*
Succinate cytochrome <i>c</i> reductase		$+32.5 \pm 7.9$ (13)*	-15.3 ± 1.8 (7)*
Succinate dehydrogenase		$+54.9 \pm 15.1$ (9)*	$+61.0 \pm 21.0$ (4)**

Table 2. *Effect of aortic constriction of the content of cytochromes *c*, *a*+*a*₃, and *b* in left ventricle of the rat*

Results are given as mean $\pm$ s.e. The numbers of determinations are given in parentheses. N.S., not statistically significant. Per cent hypertrophy was calculated from the body weight/heart weight ratios (*R*) as follows:

$\left(\frac{R_{\text{aortic constriction}}}{R_{\text{sham operation}}} \times 100 \right) - 100$. The ratios of cytochromes *a*+*a*₃ and *b* to cytochrome *c* were calculated as described in the legend of Fig. 1.

	Cytochrome <i>c</i> (nmol/g wet wt. of tissue)	Total cytochrome <i>c</i> (nmol/left ventricle)	Percent hypertrophy	Ratios	
				Cytochrome <i>a</i> / cytochrome <i>c</i>	Cytochrome <i>b</i> / cytochrome <i>c</i>
1 day after surgery					
Sham operation	39.8 ± 0.5 (19)	17.7 ± 0.3 (22)		1.26 ± 0.01 (7)	0.87 ± 0.01 (7)
	$P < 0.05$	$P \leq 0.05$	20 ± 6	N.S.	N.S.
Aortic constriction	41.5 ± 0.5 (21)	21.6 ± 0.5 (24)		1.26 ± 0.03 (8)	0.86 ± 0.04 (8)
3 days after surgery					
Sham operation	39.9 ± 0.4 (16)	18.5 ± 0.2 (22)		1.27 ± 0.02 (10)	0.87 ± 0.03 (10)
	$P \leq 0.05$	$P \leq 0.05$	33 ± 9	N.S.	N.S.
Aortic constriction	37.6 ± 0.5 (16)	22.4 ± 0.4 (18)		1.25 ± 0.03 (10)	0.92 ± 0.02 (10)

ventricular cytochrome content were obtained by relating this value to the ratios of cytochromes *c*, *b* and *a*+*a*₃ measured in partially purified mitochondria by low-temperature spectroscopy.

Cytochrome *c*

The left-ventricular cytochrome *c* content and concentrations are shown in Table 2. Cytochrome *c* content was significantly increased ($P < 0.001$) 1 day after banding of the aorta. This increase was slightly greater than the increase in mass of the heart, as is reflected in a small, but significant ($P < 0.05$) increase in cytochrome *c* 'concentration' (nmol/g wet or dry

wt.). At 3 days after aortic constriction, left-ventricular cytochrome *c* content remained greater ($P < 0.001$) than control values but the total heart mass increased to a greater extent. This is reflected by a significant ($P < 0.001$) decrease in cytochrome *c* 'concentration.'

Low-temperature spectroscopy

No attempt was made to determine the molar concentrations of the various mitochondrial cytochromes directly from the liquid-N₂-temperature spectra. Instead, the ratios of the absorption bands specific for each cytochrome were measured. To

validate the procedure, we recorded the absolute spectra of five separate mitochondrial preparations isolated from different quantities of heart, both at room temperature and at liquid N_2 temperature. Rather consistent liquid N_2 /room temperature absorption ratios of 6.5 ± 0.1 , 6.3 ± 0.2 and 8.0 ± 0.3 (mean $\pm$ s.e.m.) were observed for cytochromes *a*, *b* and *c*, respectively. Further, low-temperature spectral measurement of different dilutions of a mitochondrial preparation showed identical ratios for cytochromes *a*, *b* and *c*, and a linear increment in the absorption maxima of each cytochrome over a 3.5-fold range of concentration. A characteristic low-temperature spectrum is seen in Fig. 1.

The ratios of the relative amounts of cytochromes *a* and *b* to cytochrome *c* in aortic banded and control animals are shown in Table 2. No significant differences ($P < 0.20$) from control ratios were observed. It therefore appears that, in our present experiments,

Fig. 1. Absolute spectrum, at liquid- N_2 temperature, of partially purified mitochondria from left ventricles of two rats

The relative amounts of cytochromes *c*, *b* and *a* + *a*₃ were estimated by a modification of the method described by Claisse *et al.* (1970). Reference base-lines (· · ·) were constructed to connect the absorption minima at 540 and 570 nm and the minima at 570 and 630 nm. Base-line values were subtracted from the absorption maximum for each of the cytochromes, i.e., cytochrome *c*, 549 nm; cytochrome *b*, 560 nm; cytochrome *a* + *a*₃, 600 nm. Ratios of cytochromes *a* + *a*₃ and *b* to cytochrome *c* were calculated from these values.

changes in cytochrome *c* reflect changes in other cytochromes of the inner mitochondrial membrane.

The possibility that there was selective loss of cytochrome *c* from mitochondria of hypertrophied hearts was evaluated by measuring the amount of cytochrome *c* present in the 105000g supernatant and in the washes from mitochondrial preparations. Cytochrome *c* present in the supernatant was found to represent 1.9–4.4% of total cardiac cytochrome *c* in five separate determinations in the aortic banded and control groups. There was no difference between the two groups.

*Incorporation of δ -amino[2,3- 3H_2]laevulinate into cytochrome *c**

After intravenous administration of δ -amino[2,3- 3H_2]laevulinate, the label rapidly enters the cardiac intercellular space. The radioactivity in this cardiac free δ -aminolaevulinate (expressed as c.p.m./g wet wt. of heart) reaches a peak 5–10 min after injection

Fig. 2. Decay of radioactivity in the free δ -amino-laevulinate of rat heart

δ -Amino[2,3- 3H_2]laevulinate was given intravenously in the following dosages: 25 μ Ci/100g body wt. for the interval between 10 min and 3 h, 50 μ Ci for 6 and 12 h time-points and 200 μ Ci for 18 and 24 h time-points. Rats were killed at the times indicated and the radioactivity in the free ventricular δ -aminolaevulinate pool was determined as described in the Materials and Methods section. Results are normalized for the different amounts of radioactivity injected.

(Fig. 2). The amount of label then declines exponentially with a half-life of 15 min during the first 2 h after its administration. Between 2 and 24 h after intravenous injection the radioactivity of free δ -aminolaevulinate declines with a half-life of 3 h (Fig. 2).

The specific radioactivity of cytochrome *c* increases linearly after intravenous administration of δ -amino-[2,3- $^3\text{H}_2$]laevulinate up to 2 h, when the highest value is reached (Fig. 3). After 2 h the specific radioactivity declines exponentially with a half-life of 5.8 days (Aschenbrenner *et al.*, 1970).

Incorporation of δ -amino[2,3- $^3\text{H}_2$]laevulinate into cytochrome *c* of hearts from experimental animals 24 h after aortic constriction was about 35% greater than in sham-operated controls (Table 3). This occurred at a time when total radioactivity in the free δ -amino[2,3- $^3\text{H}_2$]laevulinate pool of both groups was identical (Table 3). Although the results suggest an increase in the rate of cytochrome *c* synthesis, definite conclusions cannot be made because it is not possible to measure the specific radioactivity of the immediate precursor of cytochrome *c* haem. Physiological changes may indeed alter the rate of entry of labelled precursor into the cell, or change the size of the precursor pool since labelling of the total free δ -aminolaevulinate pool and of cytochrome *c* are both decreased by about 50% in hearts of sham-operated rats compared with unoperated controls 24 h after surgery (results not shown).

Cytochrome *c* degradation

Experiments were designed to evaluate the contribution of changes in rates of degradation of cardiac cytochromes to the increase in mitochondrial cytochrome mass during cardiac hypertrophy. Cytochrome *c* was used as an indicator of mitochondrial cytochrome mass. In heart, cytochrome *c* has been

shown to have the same half-life as haem *a*+*a*₃ (Aschenbrenner *et al.*, 1970) and mitochondrial DNA (Gross *et al.*, 1969), and in liver the same half-life as cytochrome *b* (Druyan *et al.*, 1969) and cardiolipin (G. S. Getz, unpublished results). Therefore, cytochrome *c* is probably representative of at least a large segment of the inner mitochondrial membrane. Also, cytochrome *c* is readily extracted in adequate amounts from single rat hearts and is able to be quantified with a high degree of accuracy (Åkeson *et al.*, 1969; Williams & Thorp, 1969). δ -Aminolaevulinate is a non-reutilizable precursor and is rapidly removed from the tissues. Three separate experiments were performed, and the results

Fig. 3. Time-course of incorporation of δ -amino[2,3- $^3\text{H}_2$]laevulinate into rat ventricular cytochrome *c*

δ -Amino[2,3- $^3\text{H}_2$]laevulinate (50 $\mu\text{Ci}/100\text{g}$ body wt.) was administered intravenously and rats were killed at intervals. The specific radioactivity of ventricular cytochrome *c* was measured as described in the Materials and Methods section.

Table 3. Effect of aortic constriction on the labelling of myocardial cytochrome *c* with δ -amino[2,3- $^3\text{H}_2$]laevulinate

Results are given as mean $\pm$ S.E.M. The numbers of determinations are given in parentheses. Animals were killed 30 or 60 min after intravenous administration of δ -amino[2,3- $^3\text{H}_2$]laevulinate.

Expt.	Time after injection	...	Free δ -amino[2,3- $^3\text{H}_2$]- laevulinate (c.p.m./g of heart)	Cytochrome <i>c</i> radioactivity (c.p.m./nmol)	
				30min	60min
1 day after surgery					
	Sham operation		2880 $\pm$ 140 (9)	9.50 $\pm$ 0.8 (3)	23.5 $\pm$ 1.5 (4)
			<i>P</i> > 0.5	<i>P</i> < 0.02	<i>P</i> < 0.02
	Aortic constriction		3020 $\pm$ 125 (4)	14.00 $\pm$ 0.9 (5)	28.8 $\pm$ 1.2 (3)
3 days after surgery					
	Sham operation		5490 $\pm$ 320 (4)	18.05 $\pm$ 2.8 (2)	36.8 $\pm$ 1.2 (6)
			<i>P</i> > 0.5	<i>P</i> > 0.5	<i>P</i> > 0.5
	Aortic constriction		5536 $\pm$ 370 (3)	17.20 $\pm$ 3.0 (2)	37.5 $\pm$ 2.0 (4)

are shown separately (Fig. 4). The degree of hypertrophy attained in the three experiments was 15, 17 and 16% on day 1, and 25, 33 and 35% on day 3.

Fig. 4. Effect of aortic constriction on the degradation of cardiac cytochrome c

●, Values obtained in sham-operated rats; ○, values from rats with aortic constriction; ▲, △, the mean of each experimental group ±S.E.M. All rats received δ -amino[2,3-³H₂]laevulinate 3 days before operation with aortic constriction on day 0. In Expt. (a), label was administered into the tail vein and in Expts. (b) and (c), intraperitoneally. The total cytochrome c radioactivity was calculated by multiplying its specific radioactivity (c.p.m./nmol) by total amount of cytochrome c in the heart (nmol). The values given below are means ±S.E.M.

Cytochrome c was labelled with δ -amino[2,3-³H₂]laevulinate 3 days before banding, and total cardiac cytochrome c radioactivity (specific radioactivity × total amount of cytochrome c in the myocardium) was compared with that of sham-operated animals 1 and 3 days after surgery. No free δ -amino[2,3-³H₂]laevulinate could be detected at the time of surgery, i.e. 3 days after δ -amino[2,3-³H₂]laevulinate administration. The rate of destruction of cytochrome c can be estimated from the decline in total cardiac cytochrome c radioactivity.

It is apparent from Fig. 4 that total cardiac cytochrome c radioactivity in aortic banded animals is significantly higher than that of controls 1 day postoperatively in all experiments. Thus the rate of destruction of cytochrome c during the first post-operative day was significantly slower in experimental animals. The slope between the first and third day was not different from controls in Expts. (a) and (c). In Expt. (b), however, the rate of decrease of total cytochrome c radioactivity of the hypertrophied hearts between days 1 and 3 was slower than that of the control hearts. Thus it appears that, in this experiment, degradation was considerably decreased during the first day and may have remained slower than normal from day 1 to day 3.

An important factor that should be considered in the evaluation of these results is that hypoxia itself may lead to mitochondrial destruction (Aschenbrenner *et al.*, 1971). Hypoxia associated with surgery may affect the cytochrome c destruction rate. Our early experiments, in fact, suggested an increased rate of destruction of cytochrome c after aortic banding (Aschenbrenner *et al.*, 1972). It is likely that these results are due to our experimental design in which we attempted to resuscitate animals for prolonged periods of time. Aortic banded animals were more likely to require long resuscitation efforts and therefore to be exposed to prolonged periods of hypoxia. In the later experiments reported here, attempts were made to equalize the degree of hypoxia in aortic banded and sham-operated animals. Surgery was completed within 1–3 min, and all animals not resuscitated within 1 min after surgery were discarded.

Time after operation (days) ...	Cytochrome c (nmol/heart)		
	0	1	3
Expt. (a)			
Sham operated	26.1 ± 0.3	25.6 ± 0.2	25.8 ± 0.4
Aortic constriction	—	29.9 ± 0.3	30.5 ± 0.5
Expt. (b)			
Sham operated	—	22.6 ± 0.3	22.9 ± 0.2
Aortic constriction	—	24.8 ± 0.3	25.8 ± 0.5
Expt. (c)			
Sham operated	—	22.5 ± 0.1	22.8 ± 0.2
Aortic constriction	—	25.1 ± 0.2	26.1 ± 0.3

Discussion

Mitochondrial mass in cardiac hypertrophy

Although it is clear that myocardial mass increases rapidly in response to pressure or volume overload, the pattern of increase of individual myocardial components still has not been fully delineated. Our data, with cytochrome *c* as a marker of inner mitochondrial membrane development, indicate that there is a relative increase in mitochondrial mass 24 h after aortic constriction in the rats; but at 72 h relative mitochondrial mass has already decreased as has been generally observed in long-term pressure-induced hypertrophy (Rabinowitz & Zak, 1972; Rabinowitz, 1973). When heart enlargement develops in response to gradually imposed work-overload (McCallister & Brown, 1965; Wollenberger *et al.*, 1966), or to volume overload (Dart & Holloszy, 1969), no decrease in relative mitochondrial volume was observed. Preferential accumulation of mitochondrial proteins in the early stages of growth is further indicated by the increase in mitochondrial respiratory enzyme activities/mg of homogenate protein 24 h after aortic constriction observed in the present study. The possible role of apparent preferential mitochondrial accumulation in the pathogenesis of the hypertrophy process is still obscure.

*Turnover of cytochrome *c* in hypertrophied heart*

Although the adult cardiac muscle cell does not divide and thus may live as long as the entire organism, all of the constituents within the cell, i.e. the myofibrillar proteins (Zak *et al.*, 1971; Morkin *et al.*, 1972), the mitochondria (Rabinowitz, 1973) and membrane and soluble proteins (Young, 1970), are in a state of dynamic equilibrium, constantly being destroyed and resynthesized. We have shown in rat heart that mitochondrial DNA (Gross *et al.*, 1969), proteins and cytochromes (Aschenbrenner *et al.*, 1970) have similar half-lives of 5–6 days. Myofibrillar proteins also turn over with a half-life estimated to be between 8–10 days. It is apparent from the rapid turnover of cardiac constituents that their accumulation in response to work overload may result from a decrease in the rate of destruction as well as from an increase in the rate of synthesis. The data in the present paper indicate that this is indeed the case for cytochrome *c*.

In non-steady states the rate of degradation of a given cell component can be determined from the decline in total radioactivity of a purified component (Koch, 1962). Measurements of degradation of proteins are complicated by the reutilization of labelled amino acids derived from the destruction of myocardial proteins and the persistence of significant amounts of labelled amino acids in the cell, both of which falsely prolong the calculated half-life (Morkin

et al., 1972). Fortunately a precursor suitable for investigating the turnover of mitochondrial cytochromes is available. δ -Amino[2,3- $^3\text{H}_2$]laevulinate is specifically incorporated into the haem group of cellular cytochromes (Druyan *et al.*, 1969), and is not incorporated significantly into the protein moiety. Further, as the present paper shows, the precursor is rapidly removed from the cellular pool and results in an effective pulse-label of cytochrome *c*. Finally, the labelled haem is degraded to bilirubin and cannot be reutilized (Lester & Troxler, 1969).

In all experiments reported here total radioactivity in ventricular cytochrome *c* was significantly higher 24 h after aortic constriction than in sham-operated control rats indicating that the rate of cytochrome *c* destruction was diminished during the first 24 h after surgery. In one experiment, the slope of the curve of total cytochrome *c* radioactivity between days 1 and 3 was less gradual than in control rats, indicating a continued decreased destruction rate during this time. In the other two experiments, the slopes for experimental and sham-operated groups were not significantly different, indicating a return of the cytochrome *c* degradation rate toward normal values.

It is also likely that the rate of synthesis of cytochrome *c* is increased early after aortic constriction because overall cardiac protein and RNA synthesis increases at that time (see Rabinowitz & Zak, 1972). It may be calculated from the half-life of cardiac cytochrome *c* of 5.8 days (Aschenbrenner *et al.*, 1970) that, if degradation were completely shut off, an 11% increase in left-ventricular cytochrome *c* would occur in 24 h. This accounts for approximately 50% of the actual increment in cytochrome *c* content measured 24 h after aortic constriction. Thus an increased rate of cytochrome *c* synthesis must also be present. The 35% greater incorporation of δ -amino[2,3- $^3\text{H}_2$]laevulinate into cardiac cytochrome *c* in aortic banded animals than in sham-operated controls 24 h after surgery is consistent with this view.

Our present studies with δ -amino[2,3- $^3\text{H}_2$]laevulinate to label the haem of cytochrome *c* assume that the synthesis and degradation of the haem moiety reflect those of cytochrome *c*. In fact, the haem and apoprotein of cytochrome *c* have been shown to be synthesized co-ordinately in rat liver (Druyan *et al.*, 1973; Davidian *et al.*, 1969). Inhibition of protein synthesis promptly inhibits the incorporation of δ -amino[2,3- $^3\text{H}_2$]laevulinate into cytochrome *c*. This result contrasts with the continued labelling of the haem of cytochrome *b₅* by δ -amino[2,3- $^3\text{H}_2$]laevulinate after cycloheximide administration, a finding that suggests the presence of a *b₅* apoprotein pool, or a free haem *b* pool that exchanges with the haem of cytochrome *b₅*. Pools of cytochrome *c* apoprotein or of haem *c* do not appear to be present.

Cytochrome *c* appears to be a good indicator of changes in the other inner-mitochondrial-membrane cytochromes of the electron-transport chains, i.e. cytochrome *b*, *c* and *a+a₃*, and perhaps reflects changes in a considerable part of the inner mitochondrial membrane. Although cytochrome *c* is a soluble protein synthesized in cytoplasmic ribosomes and transported into the mitochondria (Rabinowitz & Swift, 1970), more than 95% of cell cytochrome *c* is localized in the inner mitochondrial membrane (Davidian *et al.*, 1969). Cytochrome *c* turnover rates are identical with those of other inner-membrane components (Rabinowitz, 1973). Finally, the low-temperature spectral results presented here indicate parallel changes in cytochrome *c*, *b* and *a+a₃* content during the development of cardiac hypertrophy. Thus at least under the conditions of the present experiment, changes in synthesis and degradation of cytochrome *c* probably reflect changes in the synthesis and degradation of other components of the inner mitochondrial membrane as well.

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