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Studies of Cytochrome Synthesis in Rat Liver

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The incorporation of radioactive amino acids and of δ-amino[2,3-$^3H_2$]laevulinate into rat liver cytochromes b_5 and *c* and cytochrome oxidase has been examined with and without protein-synthesis inhibitors. Cycloheximide promptly inhibits labelling of both haem and protein for cytochrome *c* in parallel fashion. Although incorporation of ^{14}C-labelled amino acid into microsomal cytochrome b_5 is also rapidly inhibited, cycloheximide incompletely inhibits haem labelling of cytochrome b_5 and cytochrome $a+a_3$, and inhibition occurs only after repeated antibiotic injections. The possibility of apoprotein pools, or of haem exchange, with a rapidly renewed 'free' haem pool, is considered. Consistent with this model is the observation of non-enzymic haem exchange *in vitro* between cytochrome b_5 and methaemoglobin. Chloramphenicol, injected intravenously over 5h, results in a 20–40% decrease in incorporation of δ-amino[2,3-$^3H_2$]laevulinate into haem $a+a_3$ and haem of cytochromes b_5 and *c*. With the dosage schedule of chloramphenicol studied, amino acid labelling of total liver protein and of cytochrome *c* was not inhibited. Similarly, ferrochelatase activity was not decreased.

δ-Aminolaevulinate is a specific haem precursor. In radioactive form, δ-aminolaevulinate has served as a site-specific label to generate haemoproteins of high specific radioactivity. Since haem is degraded to bilirubin, and is hence excreted, radioactive δ-aminolaevulinate has proved valuable in measurements of liver and muscle haemoprotein turnover, where problems associated with isotope reutilization are averted (Aschenbrenner *et al.*, 1970; Druyan *et al.*, 1969).

Alternatively, since radioactivity from δ-aminolaevulinate is not significantly incorporated into the protein moiety of haemoproteins (Druyan *et al.*, 1969) and since [^{14}C]lysine is not an efficient haem precursor, simultaneous injection of [^{14}C]lysine and δ-amino[2,3-$^3H_2$]laevulinate affords the opportunity to examine patterns of haem and protein labelling during studies of rat liver cytochrome synthesis *in vivo*. In the present paper we report studies of the synthesis of three rat liver cytochromes, in the presence and in the absence of two inhibitors of protein synthesis, cycloheximide and chloramphenicol.

Materials and Methods

Adult female Sprague–Dawley rats, each weighing 220–250g, were starved overnight before use. For cycloheximide experiments, injections were made intraperitoneally; where chloramphenicol was studied, antibiotic and radioisotopes were given intravenously. Rats were killed by decapitation; in experiments where chloramphenicol concentrations were measured, 2ml of blood was drawn from the abdominal aorta of animals lightly anesthetized with ether. Blood concentrations of chloramphenicol free base were determined by the method of Hughes & Diamond (1964).

Preparation of subcellular fractions

A 20% (w/v) liver homogenate was prepared in 0.3M-sucrose–1mM-EDTA, pH7.4, immediately after the animal was killed. Cell debris was removed by centrifugation at 600*g* for 10min and a mitochondrial pellet was prepared from the supernatant fraction by centrifugation at 12000*g* for 12min. After the pellet had been suspended in a volume of sucrose–EDTA equal to the original homogenate, the suspension was re-centrifuged at 12000*g* for 10min. Microsomal pellets were prepared as described by Druyan *et al.* (1969) except that pellets were rehomogenized in 0.15M-KCl, and were re-centrifuged at 100000*g* for 60min.

Preparation of haem $a+a_3$

Because of instability of haem $a+a_3$, exposure to light was avoided during the isolation procedure. Mitochondria were delipidated by extraction with chloroform–methanol (2:1, v/v) and then three times with acetone. Haems were cleaved from protein by extraction of delipidated mitochondria with acetone–HCl (0.9ml of 36% HCl/100ml of acetone). Haems were dried by evaporation under N_2. Haem $a+a_3$

was separated from protohaem by a modified t.l.c. technique (Schiefer, 1969). A biphasic solvent system was used: pyridine–chloroform–0.012M-$NaHCO_3$–iso-octane (20:10:10:1, by vol.) equilibrated overnight at 23°C. HHR silica gel plates (Analtech Co., Wilmington, Del., U.S.A.) were activated at 120°C for 20min, and then were cooled in an atmosphere saturated with upper-phase vapour. Duplicate 50μl haem samples, dissolved in lower phase, were applied to the silica-gel plates under N_2 and the plate was then developed for 45min in the lower phase. Well-demarcated green bands of haem $a+a_3$ were scraped off under N_2 and suspended in 1ml of pyridine. After centrifugation at 10000g for 10min, haem $a+a_3$ concentration was measured by absorption spectroscopy of the supernatant (Caughey & York, 1962). The absorption spectrum of dithionite-reduced haem $a+a_3$ is shown in Fig. 1. In several experiments, membrane cytochrome oxidase was prepared (Jacobs *et al.*, 1966). Identical specific radioactivities were obtained when haem $a+a_3$ was isolated from mitochondria or from membrane cytochrome oxidase.

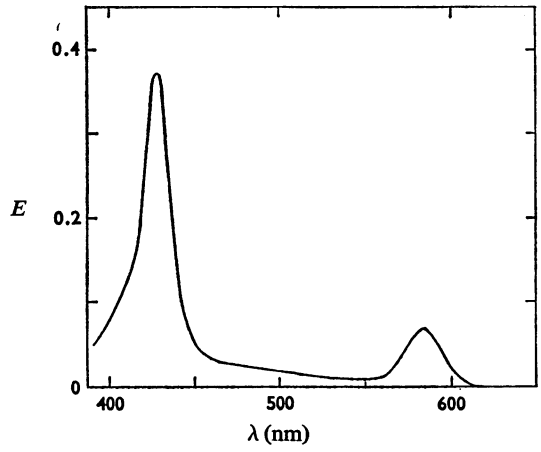

Fig. 1. *Absorption spectrum of dithionite-reduced haem $a+a_3$ isolated by t.l.c.*

Haem $a+a_3$ was dissolved in pyridine and was reduced by adding sodium dithionite, dissolved in 10μl of water.

Microsomal cytochrome b_5

The preparation and measurement of cytochrome b_5 from microsomal pellets were done as previously described, including chromatography on DEAE-cellulose (Druyan *et al.*, 1969).

Cytochrome c

Where only haem labelling by δ-amino [2,3-$^3H_2$]-laevulinate was studied, a rapid preparation method for cytochrome *c* was used (Druyan *et al.*, 1969). After injection of radioactive amino acids, a more extensive purification was performed. Four livers were pooled and a crude mitochondrial pellet was obtained by centrifugation of a 20% homogenate at 12000g for 10min. Cytochrome *c* was extracted from mitochondria with 0.15M-KCl, and was passed through a small column of Amberlite XR-64 (Druyan *et al.*, 1969). Cytochrome *c* was then applied to a column (1cm×50cm) of Sephadex G-75. Finally, cytochrome *c* was eluted from a column (1cm×10cm) of Amberlite IRC-50 by gradient elution (0–0.5M-NaCl in 0.02M-sodium phosphate buffer, pH7.2 (Margoliash & Walasek, 1967). On the basis of absorption ratios measured at 280nm (oxidized) and 550nm (dithionite-reduced), cytochrome *c* was 95–100% pure (Margoliash & Walasek, 1967).

Ferrochelatase activity

Enzyme activity was assayed as described by Jones & Jones (1969). The synthesis of both haem and cobalt mesoporphyrin IX was measured after forming the corresponding alkaline pyridine haemochro-

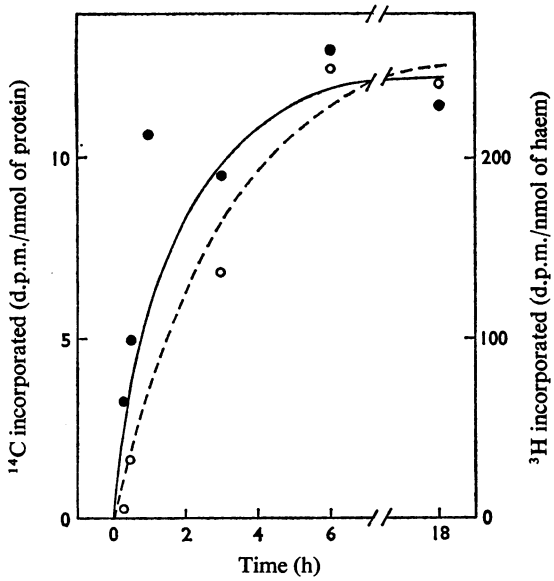

Fig. 2. *Time-course of radioisotope incorporation into cytochrome c*

At zero time, 100μCi of [^{14}C]lysine and 100μCi of δ-amino[2,3-$^3H_2$]laevulinate were injected intraperitoneally and rats were killed at intervals thereafter. For each determination, four rat livers were pooled during homogenization. ●, [^{14}C]Lysine; ○, δ-amino[2,3-$^3H_2$]laevulinate.

mogens. Mesoporphyrin IX was separated by pH and solvent extraction after hydrolysis of its dimethyl ester (Falk, 1964). Samples were incubated at 35°C for 5, 10 and 15min; Co^{2+} was omitted from blank tubes. For haem formation, samples were incubated for 90min at 35°C under a N_2 stream. Blank tubes were incubated aerobically. Yeast extract (Jones & Jones, 1969) was not added.

Analytical methods

Protein concentration was measured with the biuret (Gornall *et al.*, 1949) or Lowry (Lowry *et al.*, 1951) methods with crystalline bovine serum albumin as a standard. A Cary model 14 spectrophotometer, equipped with a dual-range slide wire, was used to record absorption spectra. Radioactivity was measured by liquid-scintillation counting, in either a Packard Tri-Carb 3365 (Packard Instrument Co., Downers Grove, Ill., U.S.A.) or a Beckman DPM-100 (Beckman Instruments Inc., Fullerton, Calif., U.S.A.) counter. Radioactive samples were counted in Scintisol Complete (Isolab Inc., Elkhart, Ind., U.S.A.). External standards were counted, and corrections were made in accordance with the manufacturer's instructions. Radioactivity results are expressed as d.p.m.

Materials

Protoporphyrin IX was purchased from Calbiochem, Los Angeles, Calif., U.S.A. Cycloheximide, bovine haemoglobin and mesoporphyrin IX dimethyl ester were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Chloramphenicol sodium succinate was obtained from Parke Davis, Detroit, Mich., U.S.A. δ-Amino[2,3-$^3H_2$]laevulinate, [$^3H_2$]-leucine, [$^3H_2$]lysine, and [^{14}C]lysine were purchased

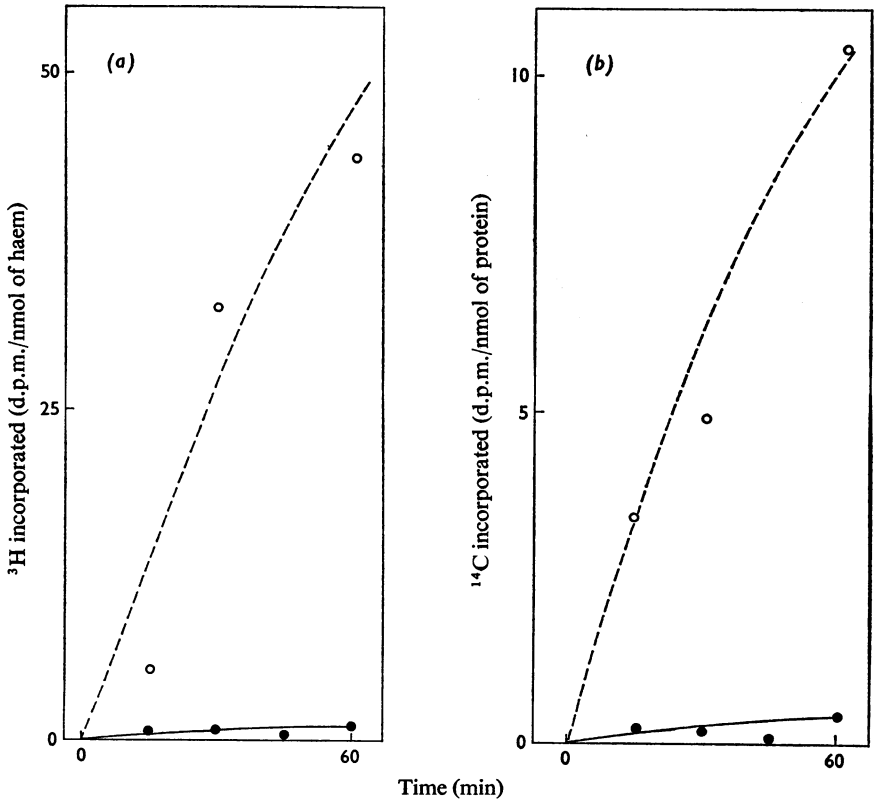

Fig. 3. *Effect of cycloheximide on radioisotope incorporation into cytochrome c*

At zero time 100μCi of δ-amino[2,3-$^3H_2$]laevulinate (*a*) and 100μCi of [^{14}C]lysine (*b*) were injected intraperitoneally. Cycloheximide (5mg/kg) was injected together with radioisotopes. ○, Control; ●, cycloheximide.

from Schwarz BioResearch, Mount Vernon, N.Y., U.S.A.

Results

Effect of cycloheximide on incorporation of precursors into cytochromes c and b_5

As shown in Fig. 2, parallel incorporation of δ-amino[2,3-$^3H_2$]laevulinate and [^{14}C]lysine into cytochrome *c* was observed in control rats. Incorporation of each radioisotope was detected within 15min, and was completed by 6h. The method used for preparation of cytochrome *c* excludes contamination by [^{14}C]lysine or δ-amino[2,3-$^3H_2$]laevulinate, since protein is dialysed and passed through Sephadex G-75 before radioactivity counting. Further, the proportion total d.p.m.:E_{550}:E_{280} was measured, and remained constant through the cytochrome *c* peak eluted from the Amberlite IRC-50 column by a phosphate gradient.

In preliminary experiments, the time-course of protein synthesis inhibition by cycloheximide was studied. When cycloheximide (5mg/kg) was injected at zero time, the 1h incorporation of [$^3H_2$]leucine or [^{14}C]lysine into total hepatic protein was 95% inhibited for 5h after cycloheximide injection.

The effect of cycloheximide (5mg/kg) injected together with δ-amino[2,3-$^3H_2$]laevulinate and [^{14}C]-lysine on cytochrome *c* labelling is shown in Fig. 3. Labelling of both the haem and protein moieties of cytochrome *c* was abolished by cycloheximide.

Similar experiments were performed with microsomal cytochrome b_5. In control animals, the labelling patterns generated after injection of δ-amino-[2,3-$^3H_2$]laevulinate and [^{14}C]lysine were similar to those observed for cytochrome *c*. However, as shown

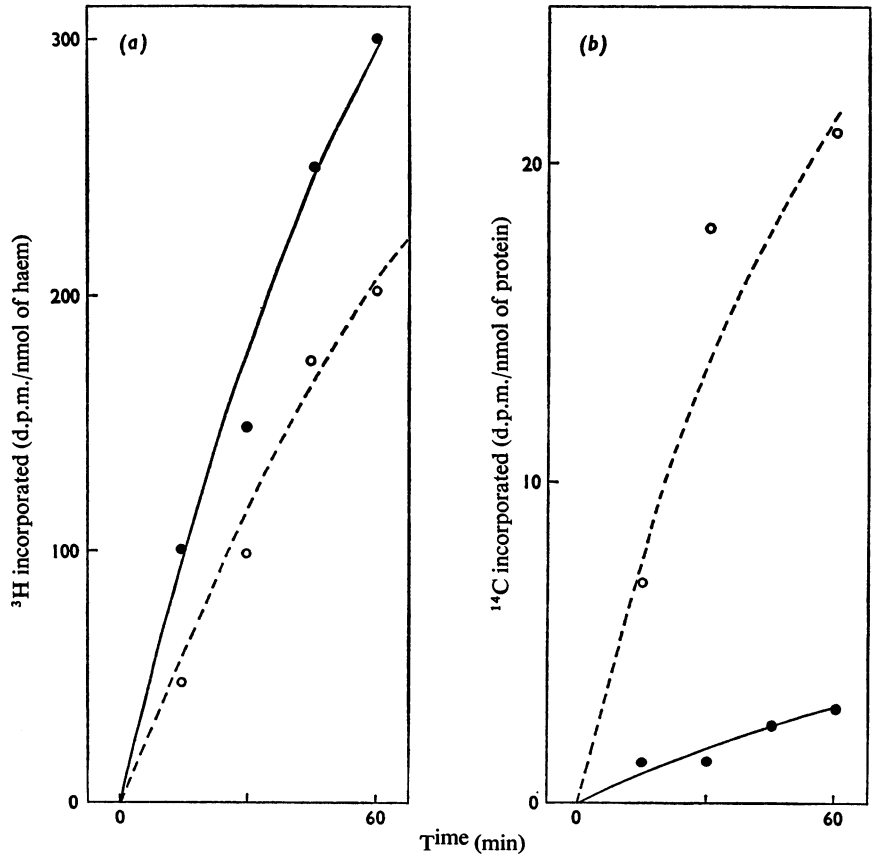

Fig. 4. *Effect of cycloheximide on radioisotope incorporation into cytochrome b_5*

Radioisotopes and cycloheximide were given as described in Fig. 3. (*a*) δ-Amino[2,3-$^3H_2$]laevulinate; (*b*) [^{14}C]lysine. Cytochrome b_5 was isolated from microsomal fractions prepared from four rat livers. ○, Control; ●, cycloheximide.

in Fig. 4, injection of cycloheximide did not inhibit haem labelling by δ-amino[2,3-$^3H_2$]laevulinate, whereas incorporation of [^{14}C]lysine into protein was blocked. As with cytochrome *c*, the preparatory technique employed for cytochrome b_5, which includes protein precipitation, dialysis and chromatography steps, excludes contamination of cytochrome b_5 by either radioactive precursor.

Haem exchange in vitro

One model that could explain the disparity between haem and protein labelling of cytochrome b_5 after cycloheximide treatment involves haem exchange; we studied haem exchange *in vitro* between cytochrome b_5 and bovine haemoglobin. Doubly labelled cytochrome b_5 was prepared from rat liver microsomal fractions 18h after injection of 50μCi of δ-amino[2,3-$^3H_2$]laevulinate and 100μCi of [^{14}C]-lysine. Non-radioactive haemoglobin was mixed together with radioactive cytochrome b_5, and the haemoproteins were separated from each other by gel filtration on a column (2.5cm×50cm) of Sephadex G-75. The specific radioactivity was measured on samples of initial and reisolated haemoproteins. The results of one experiment are summarized in Table 1, in which 37nmol of [^{3}H,^{14}C]cytochrome b_5 was incubated with 92.5nmol of unlabelled methaemoglobin (370nmol of methaemoglobin haem) for 17h at 23°C in 0.1M-sodium phosphate buffer, pH7.4. Based on ^{14}C counts, the contamination of haemoglobin by cytochrome b_5 after gel filtration was insignificant. The percentage of haem exchanged was calculated as follows: if haem exchange were complete, the specific radioactivity of all haems would be $\left(\frac{37\times440}{37+370}\right)$ = 40d.p.m./nmol. Hence, percentage exchange can be calculated as:

$$100\times\left[\frac{\text{(initial specific radioactivity)}-\text{(final specific radioactivity)}}{\text{(initial specific radioactivity)}-\text{(equilibrium specific radioactivity)}}\right]$$

In this experiment, the decrement of ^{3}H specific radioactivity for cytochrome b_5 corresponds to 79% exchange; conversely, the accretion of ^{3}H counts to methaemoglobin indicates 83% exchange.

When exchange was studied at 4°C, or with oxyhaemoglobin, haem exchange between cytochrome b_5 and haemoglobin was not detected, similar to observations reported for haem exchange between haemoglobin A and haemoglobin F (Bunn & Jandl, 1966). Several attempts were made to demonstrate haem exchange between a non-radioactive microsomal suspension and labelled cytochrome b_5 and vice versa. The results were inconclusive, however, because of incomplete recoveries.

Table 1. *Haem exchange between [^{3}H,^{14}C]cytochrome b_5 and methaemoglobin*

Doubly labelled cytochrome b_5 (37nmol) and non-radioactive methaemoglobin (92.5nmol) were mixed. After 17h, haemoproteins were separated on Sephadex G-75. Initial specific radioactivity is that before mixing cytochrome b_5 with methaemoglobin, and final specific radioactivity is that after separating incubated haemoproteins by gel filtration. Calculation of haem exchange is described in the text.

	Specific radioactivity	
Haemoprotein	^{3}H (d.p.m./nmol of haem)	^{14}C (d.p.m./nmol of protein)
Cytochrome b_5		
Initial	440	13.7
Final	122.7	10.3
Methaemoglobin		
Initial	0	0
Final	33.4	0.05

Effect of cycloheximide on the incorporation of δ-amino[2,3-$^3H_2$]laevulinate into haem $a+a_3$

Since methods available for cytochrome oxidase preparation from rat liver do not yield homogeneous proteins, only haem $a+a_3$ labelling after δ-amino-[2,3-$^3H_2$]laevulinate injection was studied. As shown in Fig. 5, cycloheximide did not inhibit haem $a+a_3$ labelling at 30min; after 60min, a 20% decrease in the specific radioactivity of haem $a+a_3$ was observed. Concomitant studies of cytochrome *c* labelling again showed immediate inhibition of haem and protein labelling by cycloheximide.

Effect of pretreatment with cycloheximide on incorporation of δ-amino[2,3-$^3H_2$]laevulinate into the haems of cytochrome oxidase, cytochromes b_5 and c

Incorporation of precursors into haem and protein moieties are dissociated by cycloheximide, suggesting that continued haem labelling, unaffected by cycloheximide, may yield holoprotein by assembly from a preformed, and hence unlabelled, apoprotein pool. To evaluate this possibility, rats were pretreated with cycloheximide for various time-intervals before measurement of 1h δ-amino[2,3-$^3H_2$]laevulinate incorporation, as shown in Fig. 6. In this experiment, rats received 2–5mg of cycloheximide, and the 1h

labelling for haem $a+a_3$, cytochromes b_5 and c was determined. For haem $a+a_3$ and cytochrome b_5, a progressive time-dependent inhibition occurred; 95% inhibition was observed at 9h for haem $a+a_3$ and 90% inhibition was found for cytochrome b_5 at 5h. For a control, cytochrome c labelling was also determined, and inhibition was almost complete at 1h.

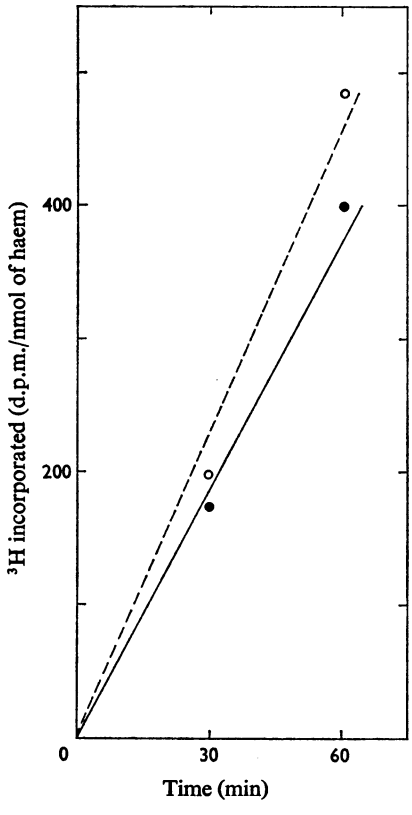

Fig. 5. *Effect of cycloheximide on haem $a+a_3$ labelling*

At zero time, 100μCi of δ-amino[2,3-$^3H_2$]laevulinate and cycloheximide (5mg/kg) were injected intraperitoneally. ○, Control; ●, cycloheximide.

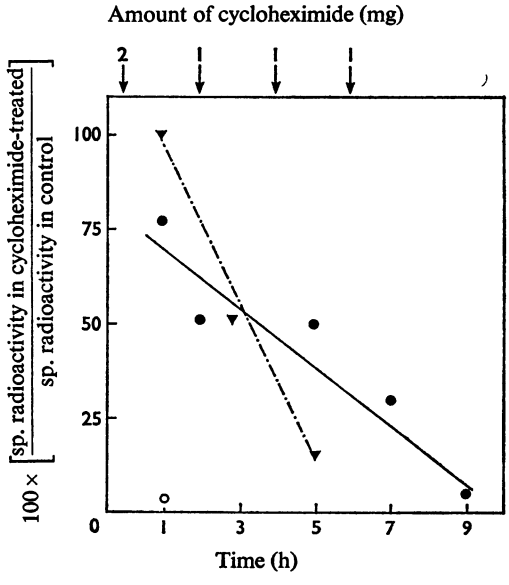

Fig. 6. *Effect of repeated cycloheximide injections on labelling of cytochrome haems*

The amount of cycloheximide injected is shown; up to 5mg was injected/rat over 6h (23–25mg of cycloheximide/kg). At various times after starting the cycloheximide injections, 100μCi of δ-amino-[2,3-$^3H_2$]laevulinate was injected and rats were killed 1h after radioisotope injection. Percentage incorporation was calculated based on specific radioactivities measured for cycloheximide-treated and control rats. ●, Haem $a+a_3$; ▼, cytochrome b_5; ○, cytochrome c.

Table 2. *Effect of chloramphenicol on the incorporation of radioactive amino acids into cytochrome c and total liver protein*

Chloramphenicol succinate was given intravenously in an initial dose of 50mg/kg, followed by 25mg/kg hourly for 4h. Radioactive amino acids were injected intravenously at 4h, and the 1h incorporation of label into purified cytochrome c and total protein was measured. N.D., not determined.

Radioisotope (dosage/rat)	Incorporation into cytochrome c (d.p.m. of ^{3}H/nmol of cytochrome c)		Incorporation into total protein (d.p.m. of ^{3}H/mg of protein)	
	Control	+Chloramphenicol	Control	+Chloramphenicol
[$^3H_2$]Lysine (200μCi)	834	908	9610	12000
(100μCi)	N.D.	N.D.	5510	6280
[$^3H_2$]Leucine (250μCi)	31.9	52.7	8060	9200
(500μCi)	62.5	97.6	26100	24400

Effect of chloramphenicol on δ-amino[2,3-$^3H_2$]laevulinate incorporation into cytochromes

Since chloramphenicol is a specific inhibitor of mitochondrial protein synthesis (Clark-Walker & Linnane, 1966), it might be expected to exert selective effects on haem $a+a_3$ labelling. To study the effects of chloramphenicol, antibiotic was given intravenously, and blood concentrations were measured before each dose: plasma concentrations were 15–25 μg/ml, a concentration sufficient to inhibit cytochrome oxidase synthesis in regenerating rat liver (Kroon & DeVries, 1969). With the doses used, no inhibition of generalized protein synthesis was observed (Table 2).

In contrast, haem labelling again showed a time-dependent inhibition, with all haems studied being similarly affected (Fig. 7). After 5h of chloramphenicol treatment, haem labelling was inhibited by 20–40% as compared with control rats. Hence, in these experiments labelling of haem $a+a_3$ was not selectively inhibited.

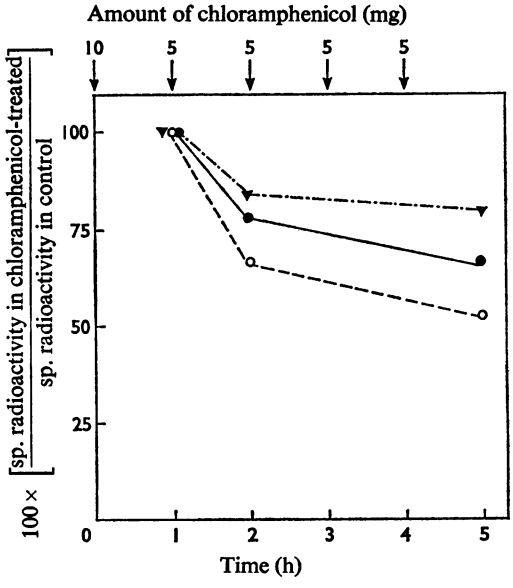

Fig. 7. *Effect of chloramphenicol on labelling of cytochrome haems*

The amount of chloramphenicol injected intravenously is shown; up to 30mg was injected/rat over 4h (138–150mg of chloramphenicol/kg). After 1, 2 and 5h, 100μCi of δ-amino[2,3-$^3H_2$]laevulinate was injected and rats were killed 1h later. Percentage incorporations, based on specific radioactivities measured for chloramphenicol-treated and control rats, were calculated. ●, Haem $a+a_3$; ▼, cytochrome b_5; ○, cytochrome *c*.

Since chloramphenicol inhibits labelling by δ-amino[2,3-$^3H_2$]laevulinate of all three haems studied, an inhibitory effect of this antibiotic on haem synthesis was suggested. Two enzymes of haem synthesis are localized in the inner mitochondrial membrane, ferrochelatase and δ-aminolaevulinate synthetase (McKay *et al.*, 1969). Although the latter enzyme is by-passed during δ-amino[2,3-$^3H_2$]laevulinate labelling, inhibition of ferrochelatase has been reported after chloramphenicol treatment (Manyan & Yunis, 1970). In rats treated intravenously with chloramphenicol for 5h we found no change in ferrochelatase activity measured by forming cobalt mesoporphyrin IX or protohaem (Table 3).

Based on measurements of incorporation of [$^3H_2$]-leucine or [$^3H_2$]lysine into total hepatic protein or cytochrome *c*, no evidence for inhibition of cytoplasmic ribosomal protein synthesis was obtained after chloramphenicol treatment (Table 2). Rather, an increased incorporation of labelled amino acids into protein was observed in most experiments, which may reflect either a decrease in the size of the cellular amino acid pool, or a true stimulation of the synthesis of a class of proteins by cytoplasmic ribosomes. Barath & Küntzel (1972) have recently reported that culture of *Neurospora* in chloramphenicol results in increased mitochondrial RNA polymerase activity. They postulate that chloramphenicol inhibits the mitochondrial synthesis of a repressor protein which is involved in the control of the synthesis of mitochondrial protein by cytoplasmic ribosomes.

Discussion

By using radioisotopic precursors which are selectively incorporated into the haem or apoprotein moieties, we have studied the synthesis of cytochrome holoproteins. For cytochrome *c*, in which haem and protein labelling are parallel, and where labelling of each is abolished by cycloheximide, synthesis is coordinated.

Table 3. *Ferrochelatase activity in mitochondria from control and chloramphenicol-treated rats*

Both haem and cobalt mesoporphyrin formation were measured by using washed mitochondria. Specific activity is expressed as nmol of product formed/h per mg of mitochondrial protein. Rats treated with chloramphenicol were killed after 5h of antibiotic treatment, injected as shown in Fig. 7.

	Cobalt mesoporphyrin	Haem
Control	4.3	2.1
+Chloramphenicol	4.5	2.0

The effect of cycloheximide clearly separates the labelling pattern of cytochrome b_5 from that of cytochrome *c*; however, our results do not permit formulation of a unique model. Since haem labelling from radioactive δ-aminolaevulinate is not blocked by cycloheximide (Levitt *et al.*, 1968), it may be inferred that δ-aminolaevulinate dehydratase and subsequent haem-synthesis enzymes are not so rapidly renewed as is δ-aminolaevulinate synthetase (Tschudy *et al.*, 1965). In an earlier report, cytochrome b_5 haem was labelled after cycloheximide treatment (Garner & McLean, 1969); we confirm that observation. The dissociation between δ-amino[2,3-$^3H_2$]-laevulinate and [^{14}C]lysine incorporation into cytochrome b_5 after cycloheximide treatment suggests two non-exclusive models. Continued haem labelling may reflect formation of holoprotein from newly labelled haem plus pre-formed, non-radioactive apoprotein. The existence of a cytochrome b_5 apoprotein pool is requisite for this model; one has been described in microsomal fractions (Hara & Minakami, 1970; Negishi & Omura, 1970).

Alternatively, haem exchange between non-radioactive cytochrome b_5 and a labelled 'free' haem pool may occur. The observation that labelled haem and labelled protein from cytochrome b_5 are degraded at different rates (Bock & Siekevitz, 1970) implies haem exchange, since the haem group of cytochrome b_5 is renewed more rapidly than the apoprotein. Further, a 'free' microsomal haem pool has been found in rats treated with δ-aminolaevulinate: the total microsomal haem concentration from treated rats was significantly greater than the sum of cytochrome b_5 plus cytochrome *P*-450 (Druyan & Kelly, 1972). Finally, we have now shown haem exchange *in vitro*, between cytochrome b_5 and methaemoglobin, in the absence of enzymic fractions. Hence, rapid cytochrome b_5 haem renewal, the existence of a microsomal haem pool and the demonstration of cytochrome b_5 haem exchange *in vitro* together suggest that haem exchange from a rapidly renewed microsomal haem pool to holoprotein cytochrome b_5 may explain our observations.

Cycloheximide exerts a similar effect on labelling of haem $a+a_3$ to that observed for cytochrome b_5. Incomplete inhibition of haem $a+a_3$ labelling was found only after repeated inhibitor injections (Fig. 5). In *Neurospora* (Weiss *et al.*, 1971) and yeast (Schatz *et al.*, 1972), some units of cytochrome oxidase are synthesized by cytoplasmic ribosomes, and their synthesis is inhibited by cycloheximide. In yeast, cytochrome oxidase apoprotein pools, synthesized on both cytoplasmic ribosomes (Chen & Charalampous, 1969) and mitochondrial ribosomes (Rouslin & Schatz, 1969; Chen & Charalampous, 1969), have been described. The presence of a cytochrome oxidase apoprotein pool may explain the time-dependent inhibition of haem $a+a_3$ labelling during repeated cycloheximide injections. In this model, inhibition of labelling occurs as the apoprotein pool is progressively depleted. Although haem $a+a_3$ could exchange in a manner analogous to that proposed for cytochrome b_5, no locus for haem $a+a_3$ has been identified other than cytochrome oxidase.

Chloramphenicol specifically inhibits mitochondrial protein synthesis in eukaryotes (Clark-Walker & Linnane, 1966). In our studies, repeated chloramphenicol injections partially inhibited haem labelling for all cytochromes measured. In contrast, incorporation of amino acids into total liver protein and cytochrome *c* was not inhibited by identical chloramphenicol doses. Hence, the delayed, parallel inhibition of haem labelling by chloramphenicol cannot be ascribed to inhibition of cytoplasmic ribosomal protein synthesis secondary to inhibition of oxidative metabolism (Firkin & Linnane, 1968).

Prolonged chloramphenicol administration results in decreased ferrochelatase activity, presumably owing to decreased enzyme synthesis (Manyan & Yunis, 1970). This effect suggests that ferrochelatase, an enzyme tightly bound to the inner mitochondrial membrane (McKay *et al.*, 1969), may be synthesized by mitochondrial ribosomes. However, during our short-term chloramphenicol experiments, ferrochelatase activity was unchanged.

The small and time-dependent inhibition of haem $a+a_3$ labelling observed during chloramphenicol treatment could reflect the presence of a cytochrome oxidase subunit pool synthesized by mitochondria (Rouslin & Schatz, 1969). However, chloramphenicol was not selective for haem $a+a_3$, and synthesis of holoprotein of both cytochromes b_5 and *c* occurs in the endoplasmic reticulum (Davidian *et al.*, 1969; Gonzalez-Cadavid *et al.*, 1971; Sargent & Vadlamudi, 1968).

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