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EFFECT OF INTRAPERITONEAL CHLORAMPHENICOL ON SOME MITOCHONDRIAL ENZYMES IN NEONATAL RATS

MIKKO HALLMAN

Department of Medical Chemistry, University of Helsinki, Finland

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Abstract—Newborn rats were given either chloramphenicol or erythromycin intraperitoneally on 4 successive days. Chloramphenicol treatment retarded the growth of the animals. The cytochrome contents of heart, liver, kidney and brain were measured. In the chloramphenicol-treated rats the tissue and mitochondrial contents of cytochromes aa_3, b and c_1 were significantly lower than in the untreated controls. However, the cytochrome c and mitochondrial cytochrome b_5 levels were not decreased. The concentration ratios of cytochromes aa_3, b and cc_1 in tissue homogenates and in isolated mitochondria were identical. Erythromycin had no effect on the cytochrome pattern.

In heart mitochondria prepared from chloramphenicol-treated animals, the cytochrome oxidase, succinate oxidase and succinate dehydrogenase activities were significantly lower than in untreated animals, however, the succinate dehydrogenase activity was largely reactivated by ATP+oligomycin. Chloramphenicol treatment did not affect the respiratory control, ADP:O ratios or the response of mitochondrial respiration to some inhibitors and uncouplers. The activities of mitochondrial glutamate, malate, α-glycerophosphate and β-hydroxybutyrate dehydrogenases and α-ketoglutarate decarboxylase were not significantly decreased by chloramphenicol treatment.

A VAST amount of experimental evidence shows that mammalian mitochondria are capable of protein synthesis.[1, 2] Only review articles are cited here. Chloramphenicol largely inhibits incorporation of amino acids into proteins of isolated mitochondria at ribosomal level.[3, 5] However, *in vitro*[3, 4] and *in vivo*[6, 45] this antibiotic has little effect on protein synthesis by nonmitochondrial ribosomes. Therefore chloramphenicol offers a possibility to study which proteins are synthesized by mitochondria *in vivo*, and to investigate the over-all control of intra- and extramitochondrial protein synthesis.

The protein-synthesizing system of isolated rat liver mitochondria incorporates amino acids mainly into the inner mitochondrial membrane.[8] After incubation of isolated mitochondria in a medium suitable for protein synthesis, the so-called structural protein contains 65 per cent of the leucine radioactivity.[9] Further, polyacrylamide gel electrophoresis reveals that many of the structural protein fractions are labelled.[10] It has also been shown that yeast cells grown in the presence of chloramphenicol no longer synthesize cytochromes a, a_3, b and c_1. However, cytochrome c synthesis is not affected.[11]

Recently, the effect of chloramphenicol on the cytochrome pattern of HeLa cells and regenerating rat liver has been described.[12, 13] In the rat, the tissue content of many mitochondrial enzymes, including cytochromes aa_3[14] and c,[15] is increased after birth. Thus, in this animal, the period following birth may be suitable for studying the physiological induction of oxidative metabolism in mammals.

The present paper describes the effects of injecting chloramphenicol into rats, beginning 0–2 hr after birth. Adequate doses of chloramphenicol caused a reduction

in the content of some mitochondrial membrane components (cytochromes aa_3, b and c_1) in heart, kidney, liver and brain homogenates and/or isolated mitochondria. In heart mitochondria, cytochrome oxidase, succinate oxidase and succinate dehydrogenase activities were also reduced.

EXPERIMENTAL

Albino Sprague–Dawley rats were given chloramphenicol sodium succinate (Orion Oy, Helsinki) or erythromycin glucoheptonate (Eli Lilly & Co., Indianapolis, Ind.) intraperitoneally. These derivatives are hydrolysed to free chloramphenicol and erythromycin in the body. Antimycin A, cytochrome c, D(−) chloramphenicol, bovine serum albumin (Fr. V), phenazine methosulphate, oligomycin and INT* were purchased from the Sigma Chemical Co., St. Louis, Mo., TMPD from Fluka AG, Buchs, NADH and ADP from C. H. Boehringer and Soehne G.m.b.H., Mannheim, ClCCP and Tricine from Calbiochem, Los Angeles, Calif., Nagarse bacterial proteinase from the Enzyme Development Corp., New York, N.Y.

Cytochromes in tissue homogenates or in isolated mitochondria were mostly estimated with a dual wavelength spectrophotometer constructed in this laboratory by Dr. I. Hassinen. A spectral band width of 3·3 nm was used at the measuring wavelength throughout. Absorption spectra of mitochondria were obtained with a Beckman DK recording spectrophotometer equipped with a special cuvette holder to balance the light paths. For practical purposes it was often necessary to store the tissue samples at −80° before the cytochrome assays. The loss of cytochromes during storage was insignificant. Before determination of the cytochromes, excess haemoglobin was washed away and the samples homogenized with a Potter–Elvehjem homogenizer and filtered through cheese-cloth. The assay medium contained 50 mM tris, 85 mM KCl, 1 mM EDTA, 3 μM rotenone and 0·1 mM malonate, pH 7·4. 1 mM KCN (at 605–630, 550–535 and 554–540), 2 μg/ml antimycin A (at 563–575) and 10 mM succinate were used to reduce the cytochromes. Reduction of cytochromes aa_3, cc_1 and b was calculated from the changes in the difference of absorbances at 605 and 630 nm ($e = 14{\cdot}0\ mM^{-1}\ cm^{-1}$),[16] 550 and 535 nm,† 563 and 575 nm ($e = 20{\cdot}0\ mM^{-1}\ cm^{-1}$)[17] respectively.

The values for cytochromes c and c_1 were calculated from the following equation:

$$\begin{cases} E^{c}_{554} \times C + E^{c_1}_{554} \times C_1 + E^{b}_{554} \times B = \Delta A_{554} \\ E^{c}_{550} \times C + E^{c_1}_{550} \times C_1 + E^{b}_{550} \times B = \Delta A_{550} \end{cases}$$

C, C_1 and B were used to indicate the concentrations of cytochromes c, c_1 and b respectively and ΔA was the change in absorbance. The following numerical values for E (absorbance coefficient) were used:

	550–535 nm	554–540 nm
c	$18{\cdot}0\ mM^{-1}\ cm^{-1}$†	$9{\cdot}3\ mM^{-1}\ cm^{-1}$†
c_1	$18{\cdot}8\ mM^{-1}\ cm^{-1}$ [18]	$10{\cdot}3\ mM^{-1}\ cm^{-1}$ [18]
b	$-2{\cdot}4\ mM^{-1}\ cm^{-1}$‡	$2{\cdot}0\ mM^{-1}\ cm^{-1}$‡

* Abbreviations used: ClCCP, carbonyl cyanide, *m*-chlorophenyl hydrazone; NADH, reduced nicotinamideadenine dinucleotide; SDH, succinate dehydrogenase; TMPD, *N*, *N*, *N'*, *N'*-tetramethyl-1,4-phenylenediamine-dihydrochloride; Tricine, *N*-tris(hydroxymethyl) methylglycine; INT, *p*-2 iodophenyl-3-p-nitrophenyl-5-phenylmonotetrazolium chloride.

† Assayed with pure cytochrome c, type III. Sigma Chemical Co.

‡ Calculated by reducing cytochrome b with antimycin A. The values were essentially similar in isolated mitochondria and tissue suspensions.

Particularly with tissue suspensions, an artificial electron shunt from cytochrome c to cytochrome b_5 was seen. Therefore the first step necessary was to reduce cytochrome b_5 with NADH (0·6 mM) in the presence of rotenone (3 μM) (at 550–535 and 554–540). In the presence of NADH, there was also a slight reduction of cytochrome c. This reduction was calculated on the basis of the relative absorbances of cytochromes b_5 and c at wavelengths 550–535 nm and 554–540 nm. When microsomal preparations from adult rats were used to reduce cytochrome b_5 in the presence of an excess of NADH, the ratio of the absorbance coefficients of cytochrome b_5 at 554–540 and 550–535 was 4·2.

Liver and kidney mitochondria were isolated as described earlier.[16] Heart mitochondria were isolated essentially by the method of Tyler *et al.*,[17] using 5 mg of Nagarse bacterial proteinase in 2·5 ml of 270 mM sucrose containing 1 mM EDTA and 10 mM unneutralized tris for 8–12 rat hearts. After homogenization in proteinase solution for 30 sec at 0°, 20 ml of 250 mM sucrose containing 1 mM EDTA and 10 mM Tricine, pH 7·3 were added, and homogenization was continued for another 30–40 sec. Before centrifugation, 20 ml of 270 mM sucrose containing 1 mM EDTA, pH 7·4, was added. Isolation was continued in the latter medium. Oxygen consumption (at 30°) was determined with a Clark-type oxygen electrode (5331 Oxygen Probe, Yellow Springs Instrument Co., Yellow Springs, Ohio). The reaction vessel (volume 0·8–1·0 ml) was constructed at the Wenner–Gren Institute, Stockholm.

The activities of mitochondrial dehydrogenases were assayed at 30° by the method of Lee *et al.*[18] The absorbance coefficient used for reduced INT was 8·0 mM^{-1} cm^{-1} at 500 nm.

Protein concentrations were determined as described earlier.[16]

Dry weight was calculated after incubating the samples to constant weight at 90°.

Concentrations of chloramphenicol were determined by the method of Levine *et al.*[19]

RESULTS

In rats, chloramphenicol is rapidly eliminated from the body, mainly by conjugation with glucuronic acid in liver microsomes.[20] The resultant glycoside is excreted chiefly in bile. The elimination of chloramphenicol is relatively slow during the first postnatal day, but increases rapidly during the following days.[21] An attempt was made to adjust the doses of chloramphenicol to the elimination rate of this drug in order to achieve effective inhibition of mitochondrial protein synthesis. Eight hr after an intraperitoneal injection of chloramphenicol (200 μg/g) the serum and tissue levels were lower than 5 μg/ml. This is not sufficient to inhibit mitochondrial protein synthesis completely.[22] Therefore, in some experiments, chloramphenicol was given four times a day (Table 2, column C). This treatment resulted in better inhibition.

Growth rate and cytochrome concentrations

Chloramphenicol treatment retarded the growth of the animals (Table 1). It had less effect on the weights of individual organs (liver, kidney and heart). However, the diminution of cytochrome content was greatest in the heart and kidney. This seems to imply that the mechanism by which chloramphenicol retards growth is different from that by which it inhibits mitochondrial protein synthesis. Chloramphenicol affected the concentrations of cytochromes aa_3, b and c_1 in every organ studied

TABLE 1. EFFECT OF CONTINUOUS CHLORAMPHENICOL TREATMENT ON THE GROWTH RATE

			20 hr		4 days		8 days	
	Fetus 20 days	0 hr	Control	Chloram-phenicol	Control	Chloram-phenicol	Control	Chloram-phenicol
Body wt. ± S.E.M. (g)	2·5 ± 0·2	5·1 ± 0·3	5·9 ± 0·4	5·6 ± 0·3	10·3 ± 0·5	7·2 ± 0·6*	16·0 ± 1·1	9·2 ± 0·9*
Liver:								
Wt. ± S.E.M. (mg)	166 ± 12	245 ± 18	232 ± 14	222 ± 10	323 ± 19	290 ± 15	452 ± 29	377 ± 21†
$\frac{\text{dry wt.}}{\text{wet wt.}} \times 100$	21·3	23·6	24·3	23·8	25·5	24·4	26·2	27·0
Kidney:								
Wt. ± S.E.M. (mg)	21 ± 2	47 ± 2	54 ± 3	52 ± 4	106 ± 7	86 ± 3†	194 ± 8	130 ± 7*
$\frac{\text{dry wt.}}{\text{wet wt.}} \times 100$	18·0	15·2	15·5	15·0	16·5	14·5	17·1	16·0
Heart:								
Wt. ± S.E.M. (mg)	10 ± 1	20 ± 1	27 ± 2	23 ± 2	63 ± 3	47 ± 2*	98 ± 4	76 ± 4*
$\frac{\text{dry wt.}}{\text{wet wt.}} \times 100$	22·0	19·8	19·6	19·9	21·5	21·2	22·8	21·9
Brain:								
Wt. ± S.E.M. (mg)	129 ± 5	212 ± 7	239 ± 6	222 ± 7	401 ± 15	309 ± 12*	602 ± 22	385 ± 19*
$\frac{\text{dry wt.}}{\text{wet wt.}} \times 100$	12·3	12·4	13·4	12·7	12·6	12·8	12·5	12·7

The first injection of chloramphenicol occurred 0–2 hr after the birth.
The doses of chloramphenicol were: 1st day 75 μg/g × 3, 2nd day 150 μg/g × 3, 3rd–4th day 200 μg/g × 3. 5th–8th day 300 μg/g × 3.
Each point represents the mean of three to six determinations.
A significant ($P < 0·01$)* or an almost significant ($P < 0·05$)† decrease as compared to the controls.

TABLE 2. EFFECT OF *in vivo* CHLORAMPHENICOL AND ERYTHROMYCIN TREATMENTS ON CYTOCHROME CONCENTRATIONS IN TISSUES OF NEWBORN RATS

	0–2 hr	4 days					
		Control	Chloramphenicol (nmoles/g dry wt.)				Erythromycin
			A		B	C	
Heart							
cyt aa_3	34·1 ± 3·2 (8)	97·0 ± 1·5 (6)	58·5 (2)		30·6 ± 3·0 (7)*	20·3 (2)	96·9 (2)
cyt b	20·9 ± 1·7 (5)	49·3 ± 4·3 (7)	43·9 (2)		19·8 ± 2·5 (5)*	11·0 (2)	46·0 (2)
cyt cc_1	40·6 ± 3·2 (7)	111·0 ± 8·4 (7)	105·3 (2)		95·1 ± 7·4 (9)	93·2 (2)	111·4 (2)
cyt c		89·0 ± 5·1 (4)			92·0 ± 8·6 (4)		
cyt c_1		56·5 ± 3·9 (4)			26·3 ± 2·9 (4)*		
Kidney							
cyt aa_3	18·5 ± 2·4 (8)	46·0 ± 6·4 (6)	23·7 (2)		14·3 ± 1·9 (6)*		45·5 (2)
cyt b	10·7 ± 1·4 (6)	27·1 ± 2·4 (6)	16·0 (2)		9·7 ± 1·1 (4)*		26·6 (2)
cyt cc_1	18·2 ± 2·3 (8)	53·2 ± 5·1 (8)	49·9 (2)		51·5 ± 4·1 (9)		51·9 (2)
cyt c		40·0 ± 2·7 (4)			42·5 ± 4·0 (4)		
cyt c_1		22·4 ± 1·8 (4)			17·2 ± 1·7 (4)		
Liver							
cyt aa_3	28·7 ± 2·2 (8)	51·8 ± 3·6 (7)	38·7 (2)		25·1 ± 2·6 (8)*	15·1 (2)	49·1 (2)
cyt b	15·7 ± 2·8 (7)	28·7 ± 2·5 (6)	22·5 (2)		14·5 ± 1·1 (6)*	9·9 (2)	28·1 (2)
cyt cc_1	28·1 ± 2·4 (8)	54·9 ± 1·6 (9)	53·0 (2)		59·4 ± 3·9 (7)	61·3 (2)	52·2 (2)
Brain							
cyt aa_1	15·5 ± 1·7 (10)	21·8 ± 3·1 (6)	18·9 (2)		11·0 ± 1·0 (9)*	9·2 (2)	21·9 (2)
cyt b	7·2 ± 1·0 (8)	10·6 ± 1·0 (6)	9·2 (2)		6·5 ± 0·7 (7)*	5·8 (2)	10·7 (2)
cyt cc_1	14·9 ± 1·8 (10)	22·6 ± 2·2 (6)	22·7 (2)		21·7 ± 1·2 (9)	23·1 (2)	22·1 (2)

The doses of chloramphenicol: Column A, 1st day 50 μg/g × 3, 2nd day 100 μg/g × 3, 3rd–4th day 140 μg/g × 3. Column B, the same as presented in Table 1. Column C, 1st day 50 μg/g × 4, 2nd day 100 μg/g × 4, 3rd–4th day 200 μg/g × 4. The doses of erythromycin: 1st–2nd day 100 μg/g × 3, 3rd–4th day 150 μg/g × 3.

Numbers in parentheses indicate the number of individual experiments.

Results are expressed as means ± S.E.M.

* A significant decrease ($P < 0.01$) as compared to the control.

(Table 2). The results were essentially similar when the content of cytochromes was expressed on the basis of total protein in the homogenate. This is in good agreement with the results obtained with yeast.[11] During the first four postnatal days, the growth rate and increase in cytochrome content were most rapid in the heart and kidney. Therefore it is in these organs that the concentrations of membrane-bound cytochromes are most likely to be affected by chloramphenicol. For similar reasons, the changes in cytochromes were smallest, although still significant, in brain homogenates (Table 2).

Cytochrome c levels were not affected by 4 days' treatment with chloramphenicol. This can be expected, since at least the apoprotein part of this cytochrome has been reported to be synthesized in the microsomal fraction and transported from there to mitochondria.[23]

Although cytochromes a, a_3, b, c_1 and c are typically mitochondrial proteins, trace amounts of cytochrome c are often found in the microsomal fraction, partly prior to transport to mitochondria, partly because of redistribution.[24] However, most of the cytochrome c present in this fraction was subtracted when the amount of cytochrome c in tissues was calculated (see Experimental). It has been reported that the nuclear membrane of thymocytes also contains a respiratory chain with cytochromes a, a_3, b, c_1 and c.[25] However, liver nuclei contain only trace amounts of cytochrome c, possibly because of redistribution of this cytochrome.[26] Accordingly, differences between the cytochrome patterns of whole homogenate and isolated mitochondria can be taken as an indication of unequal distribution of cytochromes in mitochondria with different sedimentation properties. In the present experiments the ratios between different cytochromes were similar in homogenates and isolated mitochondria (Table 3). In some experiments the complete cytochrome spectra of isolated mitochondria were recorded (Figs. 1 and 2). In Fig. 1 only the α and β bands are included, although the absorption maxima of cytochromes b and b_5 could be better resolved in the Soret region. Figure 1 indicates that the content of cytochrome b was reduced, whereas the content of cytochrome b_5, which is not considered to be a component of the respiratory chain, was not affected.

TABLE 3. RELATIVE RATIOS BETWEEN CYTOCHROMES ASSAYED FROM TISSUE HOMOGENATES OR FROM ISOLATED MITOCHONDRIA

	Mitochondria			Whole tissue		
	cyt aa_3	cyt b	cyt cc_1	cyt aa_3	cyt b	cyt cc_1
Heart						
Control	0·87	0·41	1·00	0·87	0·44	1·00
Chloramphenicol	0·31	0·19	1·00	0·29	0·19	1·00
Liver						
Control	0·99	0·60	1·00	0·95	0·52	1·00
Chloramphenicol	0·46	0·22	1·00	0·46	0·24	1·00
Kidney						
Control	0·84	0·48	1·00	0·86	0·51	1·00
Chloramphenicol	0·27	0·19	1·00	0·28	0·18	1·00

The animals were treated with chloramphenicol for 4 days as shown in Table 1.

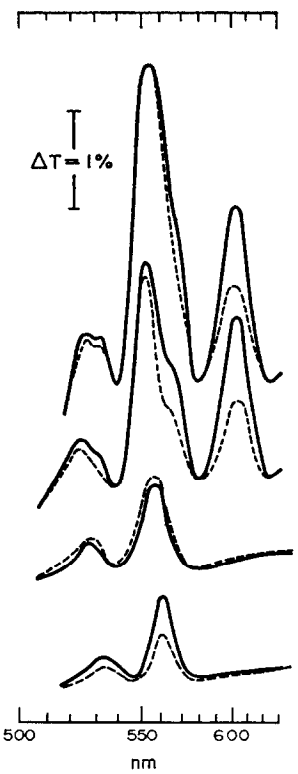

FIG. 1. Cytochrome spectra of liver mitochondria isolated from 8-day-old rats. The spectra presented are corrected for deviations of the base line. Dashed line; mitochondria from animals treated with chloramphenicol as shown in Table 1. The curves from botton to top are: (1) Cytochrome b reduced with antimycin A (2 μg/ml) and succinate (10 mM); (2) Cytochrome b_5 reduced with NADH (0·6 mM); (3) Cytochromes reduced with succinate (10 mM) and CN^- (1 mM); (4) After the assay of point 3, a few grains of dithionite were added to the cuvette. The medium contained 270 mM sucrose, 1 mM EDTA and 2 μM rotenone. The amount of mitochondria used in each assay was 2·3 mg protein/ml.

Erythromycin is known to be a specific inhibitor of mitochondrial protein synthesis in yeasts. Recently, Firkin *et al.* have shown that erythromycin has no effect on amino acid incorporation by liver mitochondria.[27] I have found the same with isolated liver and kidney mitochondria from rats of different ages (data not shown here). To confirm these *in vitro* experiments, I gave erythromycin to neonatal rats in the doses indicated in Table 2. Erythromycin had no effect on cytochrome content in the four tissues tested (Table 2).

Dehydrogenase activities and respiration of heart mitochondria after chloramphenicol treatment

Treatment with chloramphenicol for 4 days did not affect the recovery of isolated mitochondria from the tissues. The treated rats had 6·2 mg of mitochondrial protein/g of heart muscle (nine determinations), while the controls had 6·5 mg protein/g of heart muscle (seven determinations). Electron micrographs of both preparations showed well-preserved mitochondria, and only scanty amounts of smooth membranes.

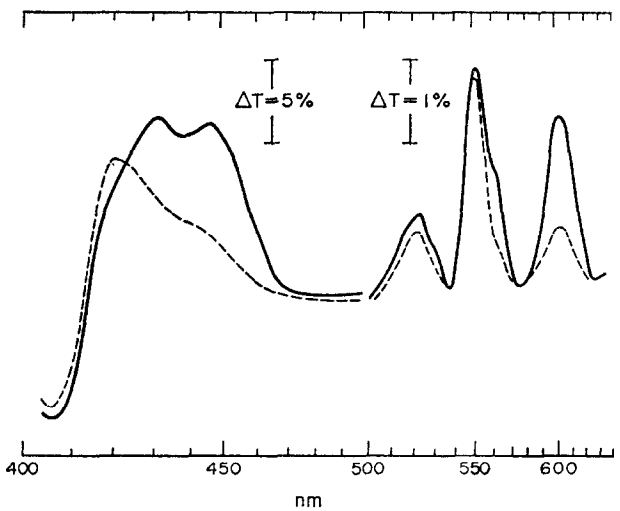

FIG. 2. Difference spectra of kidney mitochondria isolated from 8-day-old rats. Dashed line: mitochondria from animals treated with chloramphenicol for 8 days as shown in Table 1. Difference spectra were recorded after addition of succinate (10 mM) and CN^- (1 mM) to the cuvette. The amount of mitochondria was 2·5 mg protein/ml. Other conditions as in Fig. 1.

The dehydrogenase activities were determined according to the method of Lee *et al.*,[18] in which phenazine methosulphate functions as a mediator of electrons from the dehydrogenase part of the respiratory chain to INT. With succinate as substrate, INT was reduced 85–90 per cent as rapidly as oxygen (Tables 4 and 5). Changing the incubation medium to that shown in Table 5 did not increase SDH activity. However, the method used here gives reproducible values to the relative dehydrogenase activities. Of the activities assayed, only that of SDH in heart mitochondria was significantly decreased in chloramphenicol-treated animals. On the other hand, it has been reported earlier that the oxidation of succinate in mitochondria is inhibited by oxaloacetate and that this inhibition is relieved by ATP.[28, 29] To determine whether ATP has any effect on the SDH activity in heart mitochondria, some assays were made in the presence of ATP (1 mM) and oligomycin (1 μg/ml). It was found that ATP + oligo-

TABLE 4. DEHYDROGENASE ACTIVITIES IN HEART MITOCHONDRIA AFTER THE *in vivo* CHLORAMPHENICOL TREATMENT

	Control (nmoles INT reduced/mg protein/min)	Chloramphenicol (nmoles INT reduced/mg protein/min)
Succinate dehydrogenase	308·8 ± 11·3 (7)	193·3 ± 19·6 (8)*
Glutamate dehydrogenase	17·1 ± 3·0 (6)	13·9 ± 2·8 (6)
Malate dehydrogenase	32·8 ± 3·9 (7)	28·7 ± 2·0 (7)
α-Glycerophosphate dehydrogenase	10·4 ± 1·6 (5)	9·3 ± 1·1 (6)
β-Hydroxybutyrate dehydrogenase	1·4 ± 0·5 (6)	1·6 ± 0·7 (7)
α-Ketoglutarate decarboxylase	47·1 ± 4·3 (6)	40·3 ± 5·5 (7)

The animals were treated with chloramphenicol for 4 days as shown in Table 1.
Numbers in parentheses indicate the number of individual experiments.
Results are expressed as means ± S.E.M.
* A significant decrease ($P < 0·01$) as compared to the control.

TABLE 5. RESPIRATION OF HEART MITOCHONDRIA AFTER THE *in vivo* CHLORAMPHENICOL TREATMENT

	Control (natoms oxygen/mg protein/min)	Chloramphenicol (natoms oxygen/mg protein/min)	Respiratory control: Control	Respiratory control: Chloramphenicol	ADP:O ratio: Control	ADP:O ratio: Chloramphenicol
Succinate	363 ± 36 (9)	214 ± 19 (10)*	4·95 ± 0·70 (8)†	5·20 ± 0·37 (8)†	1·92 ± 0·09 (4)	1·97 ± 0·11 (4)
Glutamate-malate	117 ± 21 (4)	123 ± 10 (6)	5·37 ± 0·59 (4)†	5·91 ± 0·52 (6)†	2·98 ± 0·17 (4)	3·04 ± 0·24 (4)
Pyruvate-malate	122 ± 7 (7)	107 ± 9 (6)	6·16 ± 0·91 (7)†	6·55 ± 0·64 (6)†	3·05 ± 0·21 (4)	2·95 ± 0·22 (4)
TMPD-ascorbate	963 ± 32 (6)	489 ± 41 (8)*	1·82 ± 0·20 (4)‡	1·85 ± 0·19 (6)‡	—	—

The animals were treated with chloramphenicol for 4 days as shown in Table 1.
The assay conditions were the same as in Fig. 3.
Numbers in parentheses indicate the number of individual experiments.
The results are expressed as means ± S.E.M.
* A significant decrease ($P < 0·01$) as compared to the controls.
† Respiratory control: $\frac{O_2\text{-consumption after addition of ADP.}}{O_2\text{-consumption after exhaustion of ADP.}}$
‡ Respiratory control: $\frac{O_2\text{-consumption in uncoupled state.}}{O_2\text{-consumption in state 4.}}$

mycin increased the SDH activity significantly ($P < 0.05$) more in mitochondria obtained from the rats treated with chloramphenicol (by 35·1 per cent) than in those from the controls (by 9·5 per cent).

Mitochondrial respiration was assayed in a medium described in Table 5. When physiological substrates were present, 0·7% albumin was usually added. Albumin is known to bind free fatty acids that can uncouple oxidative phosphorylation. Accordingly, the respiratory control values were somewhat higher and more stable in the presence of albumin. However, in the assays of cytochrome oxidase, albumin was usually omitted, since some contaminant in it was found to cause a relatively high rate of auto-oxidation of ascorbate. Lowered cytochrome and succinate oxidase activities in chloramphenicol-treated rats were the only differences between the two mitochondrial preparations (Fig. 3, Table 5). No differences could be detected in the effects of rotenone (2 μM), antimycin A (1 μg/ml), dinitrophenol (0·1 mM), ClCCP (1 μM, 10 μM), oligomycin (1 μg/ml), azide (1 mM), cyanide (1 mM) and chloramphenicol (1 mM) on mitochondrial respiration. In addition, respiratory control values were essentially identical in both groups and so were the ADP:O ratios.

DISCUSSION

In the present experiments, chloramphenicol given *in vivo* was found to cause a lowered content of cytochromes aa_3, b and c_1 in all tissues studied, including the brain. This result agrees with the observations on mitochondria isolated from regenerating rat liver after chloramphenicol treatment.[13] Since extramitochondrial protein synthesis is largely resistant to chloramphenicol, these results suggest that

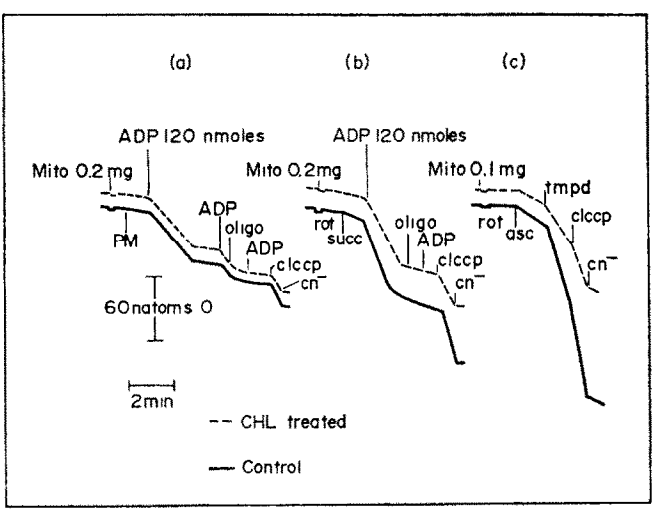

FIG. 3. Effect of chloramphenicol treatment on mitochondrial respiration. Heart mitochondria was obtained from 4-day-old rats. Dashed line: mitochondria from animals treated with chloramphenicol as shown in Table 1. (A) Respiration in the presence of 8 mM pyruvate and 8 mM malate (PM). Additions: 1 μg/ml oligomycin, 1 μM ClCCP, 1 mM CN^-. (B) Respiration in the presence of 2 μM rotenone (rot) and 10 mM succinate (succ). Additions the same as in A. (C) Respiration in the presence of 2 μM rotenone, 8 mM ascorbate (asc) and 0·6 mM TMPD. 0·5 μM ClCCP and 1 mM CN^- were added as indicated. The incubation medium contained 25 mM Tricine, 1 mM EDTA, 7 mM potassium phosphate, 100 mM sucrose, 30 mM KCl and 5 mM $MgCl_2$, pH 7·4. In addition, when succinate or pyruvate—malate were used, 0·7% bovine serum albumin was added.

the synthesis of cytochromes involves the protein-synthesizing apparatus of mitochondria. However, Gordon *et al.* have reported that amino acid incorporation into brain mitochondria and synaptosomes *in vitro*, is sensitive to cycloheximide, but resistant to chloramphenicol.[30] These results do not agree with the generally held opinion that mitochondrial protein synthesis is sensitive to chloramphenicol. Therefore more experiments with brain mitochondria isolated by different methods are needed to resolve this discrepancy.

The erythromycin resistance of protein synthesis in intact mammalian mitochondria has been confirmed[27] (Table 2). However, mitochondrial protein synthesis is sensitive to erythromycin both in *Saccharomyces cerevisiae*,[31] a facultative anaerobe, and in *Candida parapsilosis*,[32] an obligate aerobe. In addition, after mitochondrial swelling, protein synthesis in rat liver mitochondria is sensitive to erythromycin.[33] This suggests that the failure of erythromycin to inhibit protein synthesis in intact mammalian mitochondria is due to the permeability barrier at the level of the mitochondrial membrane, and not to the differences in mitochondrial ribosomes observed between yeasts and mammals.[34, 35]

Labelling experiments with isolated mitochondria suggest that the outer membrane of mitochondria,[36] cytochrome c, malate dehydrogenase,[37, 38] glutamate dehydrogenase[37] and possibly all mitochondrial proteins soluble in water or KCl (0·6 N)[39] are synthesized outside mitochondria. *In vivo* experiments also suggest that cytochrome c,[13, 24] α-glycerophosphate dehydrogenase[6] and malate dehydrogenase[13] are synthesized outside mitochondria. In the present *in vivo* studies, chloramphenicol did not decrease the contents or activities of some mitochondrial enzyme proteins (expressed on the basis of mitochondrial protein). These were cytochrome b_5 (located in the outer membrane of mitochondria), cytochrome c, glutamate-, malate-, β-hydroxybutyrate, α-glycerophosphate dehydrogenases and α-ketoglutarate decarboxylase. Together with earlier reports, the results suggest that these proteins are synthesized outside the mitochondria. If the retarded growth rate is taken into account, there is probably some decrease in the total amounts of these proteins in individual tissues. The cause of the retarded growth rate is unknown. However, 1–2 hr after injection (0·2 mg/g) the tissue concentrations of chloramphenicol were high enough to give direct inhibition of mitochondrial respiration.[21, 40] This may be one cause of the retarded growth rate.

Results both *in vivo* and *in vitro* suggest that in the liver SDH is synthesized outside mitochondria.[6, 9, 13] However, a significant inhibition of SDH activity was observed in heart mitochondria obtained from animals treated with chloramphenicol (Table 4). A different intracellular site of SDH synthesis in heart and liver seems unlikely. On the other hand, it is known that several factors can modify the activity of succinate oxidation.[29, 41] One of these factors, ATP, has recently been found to activate SDH in aged brain mitochondria.[41] In the present study the activation of SDH in heart mitochondria by ATP + oligomycin was found to be greater in chloramphenicol-treated rats than in controls. Therefore the decreased SDH activity in heart mitochondria may be due not to the decrease of its content by chloramphenicol but to an altered metabolic state of the mitochondria *in vivo* caused by the treatment with chloramphenicol.

The importance of the quantities of respiratory chain components in electron transport is poorly understood. If it was the cytochrome part of the respiratory chain

that was limiting the electron transport of cytochrome-deficient mitochondria *in vitro*, then the ratios between the activities of the dehydrogenases and the corresponding oxidases would be anomalous. The numerical ratios between succinate oxidase (state 3) and SDH in control and cytochrome-deficient heart mitochondria were 1·17 and 1·10, respectively (Tables 4 and 5). These values suggest that, at the respiratory state 3, the velocity of the over-all reaction rate between succinate and oxygen is mainly determined by the activity of SDH in both experimental groups. The situation may be different, however, when the content of the cytochromes is even more decreased.

Rapidly developing and growing tissues are particularly sensitive to inhibition of mitochondrial protein synthesis by chloramphenicol. Thus defective formation of mitochondria caused by inhibition of mitochondrial protein synthesis may be related to some of the side effects of chloramphenicol observed in human subjects, such as reversible dose-related bone marrow depression and grey syndrome[21, 42, 43] of newborn babies.

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