Differential Long-Term Effects of D-Chloramphenicol on the Biogenesis of Mitochondria in Normal and Regenerating Rat Liver

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1. Normal and partially hepatectomized rats (150g) were injected daily with D-chloramphenicol (20mg) for a period of 4 weeks, in order to investigate whether defective mitochondria could be induced *in vivo* in higher organisms as in yeast, and to measure the degree of inhibition of the mitochondrial function thus obtained. 2. The antibiotic did not affect growth and increased the amount of liver protein without changing the mitochondrial yield. 3. The respiration of isolated mitochondria from regenerated liver (regeneration completed) with succinate, α -oxoglutarate, isocitrate and malate, was decreased in the chloramphenicol-treated rats, whereas in normal liver the antibiotic increased the mitochondrial oxygen consumption with succinate and did not significantly change the respiration with other substrates. 4. Mitochondrial cytochromes and respiratory enzymes were also decreased in amount in regenerated liver from the treated rats and enhanced in normal liver. 5. The protein specific radioactivities of most mitochondrial and microsomal subfractions, 30 min after an injection of [14 C]leucine, were decreased in regenerated liver under the action of chloramphenicol. Conversely, the incorporation of [14 C]leucine into proteins of most subfractions in incubations of liver slices was enhanced in the case of normal rats treated with the antibiotic. 6. It is concluded that in regenerated liver chloramphenicol induces functionally defective mitochondria by inhibiting their biogenesis, whereas in normal liver the stimulation of respiration and protein synthesis is probably a secondary detoxication response.

The action of CAP* on mitochondrial biogenesis has been extensively studied in *Saccharomyces cerevisiae* and other micro-organisms *in vivo*. Clark-Walker & Linnane (1967) demonstrated that in the normal yeast, high concentrations of CAP in the growth medium completely inhibit the formation of cytochromes a , a_3 , b and c_1 and partially decrease succinate dehydrogenase synthesis without affecting the synthesis of cytochrome c . The number of mitochondria per cell is markedly decreased and the organelles contain few or no cristae, but apparently the formation of the outer membrane is not affected. The incorporation of radioactive amino acids into some mitochondrial fractions from derepressed yeast is also inhibited by the antibiotic *in vivo* (Henson, Weber & Mahler, 1968) and the same occurs with the activity of several mitochondrial enzymes, especially those of the inner membrane (Henson, Perlman, Weber & Mahler, 1968).

These results, together with the inhibitory effect of CAP on protein synthesis by isolated mammalian mitochondria (Rendi, 1959; Kroon, 1965; Firkin &

Linnane, 1969a), posed the question as to whether defective mitochondria could also be induced by CAP in obligate aerobic organisms *in vivo*. Kellerman, Biggs & Linnane (1969) have shown that the formation of mitochondrial cristae and cytochromes is clearly inhibited in growing *Candida parapsilosis*, an obligate aerobic yeast, in a medium containing CAP. Similarly, low concentrations of CAP block the synthesis of cytochromes a , a_3 , b and c_1 in cultures of HeLa cells (Firkin & Linnane, 1968) and substantially decrease the activity of cytochrome oxidase in beating rat-heart cells in tissue culture (Kroon & Jansen, 1968).

The production of defective mitochondria in mammalian cells by the administration of CAP to the intact animal has been reported for regenerating liver by Kroon & De Vries (1969) and by Firkin & Linnane (1969b) while the present work was in progress. Both papers coincided in showing a remarkable inhibition of the activity of cytochrome c oxidase during the first 3 days of liver regeneration. Firkin & Linnane (1969b) also demonstrated a considerable decrease in cytochromes a - a_3 , b and c_1 and some morphological

* Abbreviation: CAP: D(-)-threo-chloramphenicol.

changes in the appearance of both the cells and the mitochondria. These long-term effects of CAP obtained *in vivo* should be distinguished from the immediate inhibition of mitochondrial protein synthesis and respiration obtained in incubations of tumour cells (Haldar & Freeman, 1968) or liver slices (Beattie, 1968).

In the present work, CAP was administered to rats for 4 weeks, a period sufficiently long to assure the turning over of the whole mitochondrial population (Bergeron & Droz, 1969) under the action of the antibiotic, and the effects on mitochondrial biogenesis on normal and regenerating liver were compared. The parameters investigated were not confined to the content of cytochromes, but were extended with determinations of enzymes, respiration and protein synthesis in different mitochondrial and microsomal substructures, to obtain a more complete picture of the degree of mitochondrial impairment induced by CAP.

The results obtained indicate a clear-cut difference in the behaviour of liver tissue under the antibiotic, mitochondrial function being inhibited in the partially hepatectomized rats and stimulated in the normal rats. The first effect should be ascribed to the expected induction of defective mitochondria by CAP, whereas the other is probably a secondary response derived from a liver detoxication mechanism acting more efficiently in the normal than in the regenerating liver.

MATERIALS AND METHODS

Chemicals. The D(-)-*threo* isomer of chloramphenicol was a gift from Parke Davis, Caracas, Venezuela (sodium succinate salt). L-[U-¹⁴C]Leucine (specific radioactivity, 311 mCi/mmol) was bought from The Radiochemical Centre, Amersham, Bucks., U.K. The L-amino acids (chromatographic grade) were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. All coenzymes, substrates, detergents and chemicals used in the subcellular fractionations (except sucrose) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. All the other reagents were of analytical grade whenever available.

Treatment of animals. Male Sprague-Dawley rats from a closed colony bred at the Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela, were used throughout. Partial hepatectomy was performed by the technique described by Higgins & Anderson (1931) on 150±10 g rats, and liver regeneration was allowed to proceed to completion during the 4-week period of injections with CAP. The term regenerating liver will be applied whenever referring to changes induced during this period, and the term regenerated liver will be used when the final results of such changes are presented. The schedule of injections with CAP is explained in the legend to Table 1 and the administration of radioisotopes *in vivo* appears in Table 9. The rats were always starved overnight and killed by a blow on the head followed by decapitation, about 15 h after the last CAP injection.

Each liver was excised, cleaned of extraneous tissue, weighed, and either introduced into ice-chilled 0.25 M-sucrose for all experiments except incubations, or cut into small rectangular pieces (accumulating 3.5–4 g) and treated as explained below for the incubations.

Incubation of liver slices. The basic medium used was that of Peters & Anfinsen (1950) (containing 95 mM-NaCl, 10 mM-CaCl₂, 10 mM-KCl and 40 mM-NaHCO₃) and was freshly prepared by adding the NaHCO₃ solution to the rest of the mixture, previously gassed with O₂+CO₂ (95:5) to avoid the precipitation of CaCO₃. This saline solution was designated medium A, and was either used as such or supplemented with 20 mM-glucose alone (medium B) or 0.05 mM each of the following L-amino acids: alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

For the experiments on protein synthesis *in vitro* the pieces from a single liver were put into 30 ml of medium A in a 250 ml conical flask at 37°C, under a constant flow of water-saturated O₂+CO₂ (95:5) and were shaken at 100 oscillations/min. This was done to assure an adequate preservation of the liver pieces during the 10–15 min period demanded for cutting the slices for each sample. Slices were cut from each piece with a lucite tissue slicer, accumulated on a thin layer of 0.15 M-NaCl in a beaker, and then transferred to medium B. The washing and incubation was carried out as explained in the legend to Table 10.

For the manometric measurements each liver piece was first weighed separately and adjusted to a weight of 350–400 mg. Then, without previous incubation, the pieces were cut into slices and transferred to either new medium A or medium B as explained in the legend to Table 11. The washing and incubation are described in the same legend.

Subcellular fractionation. The incubated liver slices were separated from the medium by centrifugation (2000g, 10 min) and suspended in 30 ml of 0.25 M-sucrose with a glass rod. After being left for 15 min, the centrifugation and suspension procedures were repeated once more. Both the incubated liver slices, after washing, and the non-incubated liver, were then passed through a tissue press purchased from Climpex Ltd., London N.W.7, U.K., and made to the design of Porterfield (1960). All operations thereafter were performed at 0–4°C. The mince from each liver was suspended in 3 vol. of 0.25 M-sucrose and separately homogenized in a Potter-Elvehjem homogenizer with a tight polytetrafluoroethylene (Teflon) pestle with a clearance from the glass mortar of 0.10–0.15 mm and a diameter of 19 mm. Three up and down strokes were used over a total period of 90 s with the pestle rotating at about 1200 rev./min. The homogenates were made up to a final concentration of 10% (w/v) and then fractionated according to the procedure described by Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967a). The washed mitochondria were either used as such or subjected to subfractionation by the method of Sottocasa *et al.* (1967a), applying the 0.76–1.32 M-sucrose layers gradient (Sottocasa, Kuylenstierna, Ernster & Bergstrand, 1967b). The sonication was carried out with an MSE 100 W ultrasonic disintegrator at 8.5 μ of amplitude instead of the Branson sonifier used in the original procedure.

Microsomes were kept at -20°C and later subfractionated by the procedure of Ernster, Siekevitz & Palade (1962), by diluting the membrane fluffy layer with water and centrifuging the suspension at $105000g$ for 60 min. All pellets from the subcellular fractionations were suspended in 0.25M -sucrose with the aid of a hand-driven Potter homogenizer.

Enzyme assays. These were performed on the same day of the fractionation of the freshly prepared suspensions kept in ice until the analysis. The activities of NADH-, NADPH- and succinate-cytochrome *c* reductases were measured as described by Sottocasa *et al.* (1967a). Isocitrate dehydrogenase (EC 1.1.1.41) was assayed by the procedure of Bachmann, Allmann & Green (1966), with NADP^+ as coenzyme. Succinate dehydrogenase (EC 1.3.99.1) was measured as described by Green, Mii & Kohout (1955). The above assays were performed at 37°C in semi-micro cells of 1 cm light-path length by decreasing the respective volumes by a factor of 3. Acid phosphatase (EC 3.1.3.2), glucose 6-phosphatase (EC 3.1.3.9) and urate oxidase (EC 1.7.3.3) were measured as described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). In the first two assays, the P_i released was determined by the method of King, Abul-Fadl & Walker (1951), but the volumes were decreased by a factor of 2 and the colour was read at 750 nm. The determination of acid phosphatase was done in the presence of Triton X-100 (short incubation). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Preparation of total protein for radioactivity determinations. The protein from a sample of each fraction was precipitated with trichloroacetic acid added to 12% (w/v) final concentration. The pellets were washed twice with 5% (w/v) trichloroacetic acid and were processed by using two different procedures. The proteins from the soluble fractions and the ribosomal pellet were obtained as described by Campbell, Cooper & Hicks (1964), except that the ethanol contained 10% (w/v) potassium acetate to decrease protein losses (Munro & Downie, 1964). The

proteins from the membrane fractions were prepared by previous extraction of the phospholipids from the trichloroacetic acid precipitates with the method of Folch, Lees & Sloane-Stanley (1957). Since some proteins pass to the acidified methanol, the phospholipid extract was treated with water (0.2 vol.) and the two phases were separated by centrifugation ($2000g$, 20 min). The upper phase was discarded and the material accumulated in the interphase was pooled with the sediment from the chloroform-methanol extraction and used for protein preparation. The protein residue was then treated with 5% (w/v) trichloroacetic acid at 90°C for 20 min and thereafter processed as above for the proteins of the other sub-fractions.

The final powder was dissolved by heating in concentrated formic acid (AnalaR grade) for 30 min at 90°C (water bath) to a final concentration of approx. 10mg/ml . The exact concentration was determined by plating 0.2 ml portions on to pre-weighed 7cm^2 aluminum discs, drying under an infrared lamp and weighing.

Determination of radioactivity. Samples (0.2 ml) of protein solution in formic acid were mixed with 10 ml of the scintillation fluid 'tT21' proposed by Patterson & Greene (1965), composed of 2 vol. of toluene containing 0.4% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyloxazol-2-yl)benzene, and 1 vol. of Triton X-100. The radioactivities of the samples were counted in an automated liquid-scintillation spectrometer (Nuclear-Chicago model 720) under conditions where the ^3H efficiency was less than 0.1%. Efficiencies were determined for each sample by internal standardization and all counting was done to a 1.5% statistical error at 68% probability.

Other determinations. The determination of the rate of mitochondrial respiration was carried out with a Clark-type oxygen electrode (Beckman Instruments Inc., Fullerton, Calif., U.S.A.) as detailed in the legend to Table 3. Respiration of tissue slices was measured manometrically under conditions similar to those used for the experiments on protein synthesis, as explained in the legend to Table 4.

Table 1. *Body weight and amount of liver protein in rats treated with D-chloramphenicol*

Normal and partially hepatectomized rats ($150 \pm 10g$ body wt.) were daily injected intraperitoneally with one single dose of 20 mg of CAP in 1 ml of 0.15M -NaCl for a period of 4 weeks. The rats were starved overnight and killed around 15 h after the last injection. Values are expressed as averages $\pm$ s.e.m., except in cases with less than five determinations, where the range of values is given. The number of experiments appears in parentheses.

Rats	Body wt. (g)	Liver wt./body wt. ratio (g/100g)	Liver protein/body wt. ratio (g/100g)	Recovered pure mitochondrial protein/liver protein* ratio (%)
Normal				
Controls	255 ± 17 (18)	3.1 ± 0.4 (18)	0.53 ± 0.05 (3)	9.0 ± 0.9 (8)
Injected with CAP	260 ± 21 (12)	3.6 ± 0.4 (12)	0.72 ± 0.06 (3)	8.5 ± 0.9 (4)
Partially hepatectomized				
Controls	272 ± 20 (8)	3.1 ± 0.5 (8)	0.49 ± 0.04 (2)	7.8 ± 0.8 (8)
Injected with CAP	266 ± 21 (9)	3.6 ± 0.5 (9)	0.63 ± 0.06 (2)	7.5 ± 0.7 (8)

* The connective tissue and other debris left as residue in the tissue mincer was not included in the liver homogenates.

RESULTS

Effects of chloramphenicol on mitochondrial respiration and cytochromes. The long-term administration of CAP did not affect growth and promoted, both in normal and in partially hepatectomized rats, a slight increase of the liver wt./body wt. ratio (Table 1). The protein content of the liver was also augmented and as a result there was an increase in the liver protein/body wt. ratio of about 30%. The

proportion of mitochondria in the liver did not seem to change significantly, if judged from the yield of pure mitochondrial protein related to the total protein of the liver.

The mitochondria prepared from normal liver by the procedure of Sottocasa *et al.* (1967a) were only approx. 30% of the total population, as judged from data on the total amount of liver mitochondria given by Baudhuin & Berthet (1967) and by applying a correction to compensate for our amount

Table 2. *Assessment of the purity of the mitochondrial fraction from normal liver with marker enzymes*

The incubation of the liver was performed as for the experiments of protein synthesis (see Table 10). Values are means from two experiments.

Subcellular structure	Marker enzyme	Sp. activity (nmol/min per mg of protein)				Protein from the respective subcellular structure contaminating the mitochondria (% of protein in the mitochondrial fraction)				
		In the respective purified fraction	In the mitochondrial fraction				Non-incubated			
			Non-incubated		Incubated		Non-incubated		Incubated	
			Control	CAP	Control	CAP	Control	CAP	Control	CAP
Lysosomes	Acid phosphatase*	646†	86	77	80	68	13	12	12	10
Peroxisomes	Urate oxidase	595†	0.2	0.1	3.8	2.9	<0.1	<0.1	0.6	0.5
Microsomes	Glucose 6-phosphatase	330‡	34	37	23	26	10	11	7	8
Microsomes	NADPH-cytochrome c reductase	75‡	4	2	7	4	5	3	9	5

* Total activity, in the presence of Triton X-100.

† Values calculated from data presented by Leighton *et al.* (1968) for the purified organelles.

‡ Values determined in our microsomal fraction (controls).

Table 3. *Effect of treatment with D-chloramphenicol on mitochondrial respiration*

Liver mitochondria were obtained as described by Sottocasa *et al.* (1967a). Protein concentrations were adjusted to 20mg/ml of 0.25 M-sucrose and 0.4ml samples were used for polarographic determinations in 8.2ml of medium (0.225 M-sucrose; 10mM-MgCl₂; 5mM-KCl; 10mM-potassium phosphate buffer, pH 7.4; 20mM-triethanolamine, pH 7.4). Estabrook's (1967) technique was followed, and substrates and ADP to 6 and 0.4mM final concentrations respectively were added. Values are means of duplicate determinations from the number of experiments shown.

	Number of experiments	Respiratory rate in the active state (nmol of O ₂ /min per mg of protein)		CAP/control ratio
		Control	CAP-injected	
Mitochondria from normal liver				
Succinate	5	42.8	62.5	1.46
Isocitrate	3	27.3	27.6	1.01
α-Oxoglutarate	3	34.5	33.0	0.96
Malate	3	11.5	10.1	0.88
Mitochondria from regenerated liver				
Succinate	3	57.8	39.3	0.63
Isocitrate	2	10.9	6.2	0.57
α-Oxoglutarate	2	6.9	4.8	0.70
Malate	2	8.5	4.6	0.54

of contamination. The degree of purity calculated from the specific activities of some marker enzymes was acceptable for our purposes (Table 2). The

Table 4. *Effect of D-chloramphenicol on the amount of mitochondrial cytochromes*

The determinations were done by direct spectrophotometry of mitochondrial suspensions, as described by Williams (1964, 1968). The results are averages of two experiments.

	Cytochrome content (nmol/mg of mitochondrial protein)		CAP/control ratios
	Control	CAP-injected	
Normal liver			
Cytochrome <i>a-a</i> ₃	0.26	0.35	1.35
Cytochrome <i>b</i>	0.28	0.30	1.07
Cytochrome <i>c</i> ₁	0.16	0.20	1.25
Cytochrome <i>c</i>	0.20	0.25	1.25
Total	0.90	1.10	1.22
Regenerated liver			
Cytochrome <i>a-a</i> ₃	0.35	0.27	0.77
Cytochrome <i>b</i>	0.40	0.25	0.62
Cytochrome <i>c</i> ₁	0.15	0.14	0.94
Cytochrome <i>c</i>	0.20	0.22	1.10
Total	1.10	0.88	0.80

main contaminants were lysosomes (around 13%) and microsomes (4–10%, according to the marker enzyme). The incubation of the liver under conditions used for the experiments with radioactivity did not affect the mitochondrial purity.

Mitochondrial respiration was differentially affected by CAP, since in normal rats the respiratory rate in state 3 with 6mM-sodium succinate as substrate was remarkably enhanced (+46%) whereas in mitochondria from regenerated liver it was decreased by 37% (Table 3). On the other hand, no significant variation induced by CAP was observed in the respiration of normal mitochondria with isocitrate, α -oxoglutarate and malate, as opposed to a 30–45% decrease in mitochondria from regenerated liver. In normal liver mitochondria the respiratory control indices varied between 2.0 and 3.7, and the P/O ratios were 1.4 (succinate), 3.2 (isocitrate), 3.0 (α -oxoglutarate) and 2.7 (malate). No significant changes were observed in the CAP-treated rats. The control indices in the mitochondria from regenerated liver were low (1.4–1.8) and therefore the P/O ratios were not determined. The effects induced by CAP in mitochondrial respiration were paralleled by changes in the amount of mitochondrial cytochromes. Table 4 shows that in normal liver the total cytochromes were increased by 22%, against a 20% decrease observed in mitochondria from regenerated liver.

Table 5. *Influence of sonication on mitochondrial subfractionation*

Liver mitochondria from 260 g Sprague–Dawley rats were disrupted by hypo-osmotic swelling and shrinking with ATP, followed or not by ultrasonication, as indicated by Sottocasa *et al.* (1967a). The fractionation was done according to Sottocasa *et al.* (1967b). Our results are means from two experiments. Values in parentheses are taken from the work of Sottocasa *et al.* (1967a) on liver mitochondria from 150–200 g Wistar rats.

Disrupted mitochondria	Distribution (% of sum of fractions)			
	Protein	Succinate-cytochrome <i>c</i> reductase	NADH-cytochrome <i>c</i> reductase (rotenone-insensitive)	Isocitrate dehydrogenase
Fractions				
(a) No sonication				
Soluble	16 (10)	0 (—)	5 (0)	42
Light	8 (3)	8 (—)	44 (9)	4
Heavy	76 (87)	92 (—)	51 (91)	54
(b) After sonication				
Soluble	52 (34)	3 (0)	43 (15)	80
Light	15 (9)	24 (4)	49 (75)	0
Heavy	33 (57)	73 (96)	8 (10)	20
Sum of fractions		Sp. activity (nmol*/min per mg of protein)		
(a) No sonication	—	277 (—)	236 (393)	130
(b) After sonication	—	140 (251)	242 (366)	144

* nmol of cytochrome *c* (succinate- and NADH-cytochrome *c* reductases) or NADP⁺ (isocitrate dehydrogenase).

Purity of the mitochondrial subfractions. Table 5 shows that if mitochondria from normal rats are disrupted simply by hypo-osmoticity and ATP-mediated contraction, without sonication, the release of protein from the heavy fraction is slightly higher in our case (250g Sprague-Dawley rats) than in the case of Sottocasa *et al.* (1967a) (150–200g Wistar rats). The fragmentation is remarkably enhanced when treating with ultrasound in the MSE 100 (8.5 μ) as compared to the Branson sonifier (3A). The age of the rats does not seem to be responsible for the low yield of the heavy fraction, since this was obtained with our conditions of sonication even when 150g rats were used. For the rest of the present work only mitochondria from 250–280g Sprague-Dawley rats were used and the disruption was always carried out with sonication. According to the Sottocasa *et al.* (1967a) interpretation of the marker enzymes, our heavy fraction may be identified as reasonably pure inner membrane and the soluble fraction is mostly matrix without electron-transport-chain activities but with some material solubilized from the outer membrane. However, considering that the light fraction has nearly fourfold less protein than the soluble fraction, the magnitude of the contamination of the matrix is acceptable. The light fraction is outer membrane entirely free of matrix but with a substantial amount of electron-transport-chain activity. It can be calculated that around 40% of the light fraction is due to fragments of the inner membrane.

Effect of chloramphenicol on mitochondrial en-

zymes. When this scheme of subfractionation (with the MSE sonifier) was applied to mitochondria obtained from regenerated liver, the distribution of protein was more similar to the values of Sottocasa *et al.* (1967a), but the fragmentation of the inner membrane was still remarkable (Table 6). The rats treated with CAP showed a 35% decrease in the specific activities of succinate-cytochrome *c* reductase and succinate dehydrogenase in the sum of fractions, which agreed well with the impairment of succinate oxidation observed polarographically (–37%). Another electron transport activity, the rotenone-sensitive NADH-cytochrome *c* reductase, was considerably decreased in the treated rats (–42%). The distribution of isocitrate dehydrogenase and rotenone-insensitive NADH-cytochrome *c* reductase resembled that obtained for normal mitochondria, but the percentage retained in the heavy fraction was higher. Treatment with CAP did not significantly affect this distribution but induced a 29% decrease in the activity of isocitrate dehydrogenase. This clear inhibition of some key respiratory enzymes contrasted with the stimulation of microsomal enzyme activities, such as rotenone-insensitive NADH-cytochrome *c* reductase (+53%) and NADPH-cytochrome *c* reductase (+80%).

The effect of treatment with CAP on mitochondrial enzymes from normal liver was not studied, but some indication was gained from the analysis of mitochondria obtained from tissue slices previously incubated under the same conditions used for the experiments of protein synthesis *in vitro*.

Table 6. *Effect of D-chloramphenicol on the distribution of protein and marker enzymes among mitochondrial subfractions from regenerated liver*

Mitochondria were fractionated according to Sottocasa *et al.* (1967a,b). Values are means from seven experiments (protein) or two experiments (enzymes).

Disrupted mitochondria	Distribution (% of sum of fractions)									
	Protein		Succinate-cytochrome <i>c</i> reductase		Succinate dehydrogenase		NADH-cytochrome <i>c</i> reductase (rotenone insensitive)		Isocitrate dehydrogenase	
	Control	CAP	Control	CAP	Control	CAP	Control	CAP	Control	CAP
Fractions										
Soluble	38	35	2	3	4	10	38	40	53	68
Light	10	9	16	31	16	38	39	45	1	1
Heavy	52	56	82	66	80	52	23	15	46	31
Sp. activity (nmol*/min per mg of protein)										
Sum of fractions	—		215	140	38	25	175	193	142	101

* nmol of 2,6-dichlorophenol-indophenol (succinate dehydrogenase), and see Table 5.

Table 7. *Effect of incubation of slices of normal liver on the distribution of protein and marker enzymes among mitochondrial subfractions*

The assays were done on samples from the experiments with [¹⁴C]leucine (see Table 10). Values are means from five experiments (protein) or two experiments (enzymes). For values of non-incubated samples see Table 5.

Distribution (% of sum of fractions)										
Disrupted mitochondria	Protein		Succinate-cytochrome c reductase		Succinate dehydrogenase		NADH-cytochrome c reductase (rotenone-insensitive)		Isocitrate dehydrogenase	
	Control	CAP	Control	CAP	Control	CAP	Control	CAP	Control	CAP
	Fractions									
Soluble	39	40	2	2	7	5	7	8	79	84
Light	22	14	57	54	72	68	41	54	3	2
Heavy	39	46	41	44	21	21	52	38	18	14
Sp. activity (nmol*/min per mg of protein)										
Sum of fractions	—		90	125	32	38	150	169	101	95

* See Tables 5 and 6.

These determinations were done in order to investigate whether the incubation of the liver affected the subfractionation of mitochondria. Results shown in Table 7 demonstrate that the incubation decreased the release of protein from the inner membrane (compare with Table 5) but induced a considerable transfer of enzymes to the other fractions. The NADH-linked reductase was much less solubilized than in the case of the non-incubated normal liver and the distribution of the isocitrate dehydrogenase was not affected. This means that, on a protein basis, the degree of cross-contamination was quite similar to that obtained before incubation. Both the succinate-cytochrome c reductase activity (+39%) and the succinate dehydrogenase (+19%) activity were increased by CAP, in agreement with the stimulated mitochondrial respiration with succinate (+46%).

The subfractionation of microsomes, both in non-incubated regenerated liver and in incubated normal liver, did not show any significant difference between the controls and the CAP-treated rats (Table 8). The differences in regenerated and normal liver in the distribution of protein among the subfractions probably reflect both the physiological state of the liver and the incubation treatment.

Effects of chloramphenicol on protein synthesis. Table 9 shows that in the case of regenerated liver, the protein specific radioactivities, 30 min after the injection of [¹⁴C]leucine, were significantly decreased in the CAP-treated rats, and the inhibition was evident in all the subfractions except the ribosomal pellet. The protein specific radioactivity of the

Table 8. *Subfractionation of the microsomal fraction from regenerated liver and incubated slices from normal liver*

With incubated slices from normal liver, the assays were done on samples from the experiments with [¹⁴C]leucine (see Table 10). Microsomes were subfractionated according to Ernster *et al.* (1962). Values are means from five experiments.

Microsomal subfractions	% distribution of protein			
	Non-incubated regenerated liver		Incubated normal liver	
	Control	CAP	Control	CAP
Soluble	85.6	85.4	50.0	48.1
Membranes	7.1	9.8	5.9	6.6
Pellet	7.3	4.8	44.1	45.3

microsomal membranes was the more inhibited by CAP whereas that of the light mitochondrial subfraction was the less affected. The same effects were noticed throughout a whole time-curve of incorporation from 7.5 to 60 min (N. F. González-Cadavid & J. L. Ramírez, unpublished work).

In the case of normal rats the study of protein synthesis was carried out *in vitro* instead of *in vivo*, but as previously demonstrated the incubation did not affect either the purity of the mitochondrial fraction or the interpretation of the subfractionation. Table 10 shows that, in a 30 min incubation period with [¹⁴C]leucine, the protein specific radioactivities

Table 9. *Effect of D-chloramphenicol on the synthesis in vivo of mitochondrial and microsomal proteins from regenerated liver*

Rats were injected through the tail vein with 5 μ Ci of [14 C]leucine (320 mCi/mmol) in 1 ml of 0.15 M-NaCl. The animals were killed after 30 min and the livers fractionated as described in the text. Values are means from two experiments.

	Specific radioactivity (d.p.m./mg of protein)		CAP/control ratios
	Control	CAP-injected	
Mitochondrial subfractions			
Soluble	148	44	0.30
Light	150	93	0.62
Heavy	179	94	0.54
Sum of fractions	164	76	0.46
Microsomal subfractions			
Soluble	421	286	0.68
Membranes	498	133	0.27
Pellet	81	149	1.84
Sum of fractions	401	264	0.66

Table 10. *Effect of treatment with D-chloramphenicol on the synthesis in vitro of mitochondrial and microsomal proteins by slices from normal liver*

Liver slices (3.5–4 g) were washed in 30 ml of the medium described by Peters & Anfinsen (1950), supplemented with 20 mM-glucose, in 250 ml flasks, at 37°C, under O₂+CO₂ (95:5) for 30 min. The medium was decanted and replaced by a fresh one containing, besides glucose, natural L-amino acids (see text) and 0.166 μ Ci/ml of L-[U- 14 C]leucine (320 mCi/mmol). After 30 min leucine (10 mg in 1 ml of 0.15 M-NaCl) was added and the samples were chilled to 0°C. The fractionation and the determination of radioactivity were done as described in the text. Values are means from two experiments.

	Specific radioactivity (d.p.m./mg of protein)		CAP/control ratios
	Control	CAP-injected	
Mitochondrial subfractions			
Soluble	96	136	1.42
Light	295	214	0.73
Heavy	117	201	1.72
Sum of fractions	144	189	1.31
Microsomal subfractions			
Soluble	1372	1886	1.37
Membranes	557	1777	3.19
Pellet	360	583	1.62
Sum of fractions	874	1294	1.48

Table 11. *Effect of treatment with D-chloramphenicol on respiration of normal liver*

Slices from normal rat liver (350–400 mg) were washed in 4 ml of medium of Peters & Anfinsen (1950), supplemented or not with 20 mM-glucose, in 25 ml flasks, at 37°C, under O₂+CO₂ (95:5) for 30 min. The slices were transferred to Warburg flasks and incubated in 4 ml of fresh medium containing natural L-amino acids (0.05 mM each, see text) and supplemented or not with 20 mM-glucose, at 37°C under pure O₂. Readings were taken every 5 min for 60 min, and values are means of three determinations taken in the straight part of the plot (at least 40 min) of O₂ uptake against time. The respiration of liver homogenates (10%, w/v, in 0.25 M-sucrose) was determined polarographically, either as in Table 3 (a), or with Krebs–Ringer-phosphate (b). Values are means from two determinations.

	Respiratory rate (nmol of O ₂ /min per mg of protein)		CAP/control ratios
	Control	CAP-injected	
Liver slices			
Glucose	3.57	4.83	1.35
No glucose	2.25	1.08	0.48
Liver homogenates			
Glucose (a)	2.0	2.2	1.10
Succinate (a)	10.2	10.6	1.04
Succinate (b)	19.0	19.0	1.00

were significantly enhanced in the CAP-treated rats, and the stimulation was noticed in all the subfractions except the light mitochondrial fraction. This inhibition in the outer membrane-rich fraction contrasted with the remarkable increase in the specific radioactivity of the microsomal membrane proteins. Similar effects were noticed at 15 and 60 min (N. F. González-Cadauid, E. M. A. Bello & J. L. Ramírez, unpublished work).

In order to investigate whether there was any change in the respiration of the tissue slices from the CAP-treated normal rats under the conditions of our incubations, respiration was measured manometrically, either in the presence or the absence of 20 mM-glucose. With the glucose-supplemented medium the liver slices from the CAP-injected animals respired 35% more efficiently than those of the controls (Table 11). Since the experiments with [14 C]leucine were carried out in the presence of 20 mM-glucose, this stimulation of respiration is probably related to the observed increase in protein synthesis. The absence of glucose decreased the respiratory rate in the controls and especially in CAP-treated rats. Homogenization of the liver considerably decreased the stimulation of respiration induced by CAP in the presence of glucose. No

differences were found in the respiratory rates of liver homogenates with 6mM-sodium succinate.

DISCUSSION

In the present work we have inhibited mitochondrial biogenesis in regenerating liver *in vivo*, by simply injecting a single daily dose of 80–120mg of CAP/kg body wt. during a period sufficiently long to assure a complete tissue regeneration. This means that the alterations induced by the antibiotic are maintained well after the period of active tissue proliferation, and in a stage when the liver function should be practically restored (Higgins & Anderson, 1931; Harkness, 1957). Kroon & De Vries (1969) claimed that the necessary concentration of CAP in the blood to inhibit the synthesis of cytochromes oxidase (5–10 $\mu\text{g}/\text{ml}$) could be achieved only by subcutaneous implantation of dialysis bags containing the antibiotic, and not by injections. Firkin & Linnane (1969b), using double our daily doses (200mg/kg) in four 50mg injections, showed on the other hand that CAP was present intermittently in the serum, and the effect on cytochromes was practically negligible 48 and 72h after the operation. The differences are most probably due to a saturation of the detoxifying mechanisms of the liver during our prolonged treatment. We have obtained a 20% inhibition of the synthesis of mitochondrial cytochromes but, in contrast to Firkin & Linnane (1969b), the 40% decrease was in cytochrome *b* instead of *a-a₃*, and the increment of cytochrome *c* was not so remarkable. However, investigation of other parameters showed a more significant inhibition of mitochondrial function, as inferred from the decreases in O_2 uptake, respiratory enzymes and, especially, protein synthesis. The fact that this considerable block of mitochondrial protein synthesis is not accompanied by a significant decrease in mitochondrial yield could mean that protein synthesis is arrested only in the last stages of the treatment and therefore it cannot be reflected in a change in the mitochondrial population, of relatively slow turnover.

The inhibition of microsomal protein synthesis, especially that of the membranes, is probably, as assumed for mitochondria, a late effect, because if not it would be difficult to reconcile with the increase of microsomal enzyme activity and of total liver protein. During the 4-week treatment the regenerating liver partially compensated the inhibition of mitochondrial biogenesis and tissue respiration by increasing the number of cells, but failed to restore the full activity of mitochondria and this marks a clear distinction from the case of normal liver, as discussed below.

Firkin & Linnane (1969b) demonstrated that in non-hepatectomized rats, a 48h 600mg of CAP

regime, which maintains the concentration in the serum above 70 $\mu\text{g}/\text{ml}$, produced no detectable effect on the cytochromes of the liver mitochondria. With our treatment, we observed significant changes that were opposed to those expected from the well-known inhibitory action of CAP and the effects demonstrated in regenerated liver. Thus, the mitochondria from the animals treated with the antibiotic exhibited a higher respiratory rate with succinate, in contrast with the situation observed in the partially hepatectomized rats and with the fact that CAP inhibits the respiration of rat liver mitochondria *in vitro* (Beattie, 1968). The increase in the content of cytochromes differs from the decrease obtained both in yeast and higher organisms *in vivo* and the same occurs with the remarkable stimulation of protein synthesis *in vitro* shown by our results. This last effect is probably related to the CAP-induced higher respiratory rate of liver slices incubated in the presence of glucose. It may derive from a selective increase in membrane permeability towards certain substrates by the action of the drug, since in the absence of glucose there is inhibition instead of stimulation, which agrees with the results obtained in mouse ascites tumour cells (Haldar & Freeman, 1968) and in HeLa cells (Firkin & Linnane, 1968). Moreover, the disruption of the liver plasma membrane eliminates the stimulation.

The increase of microsomal protein synthesis, and particularly that of the membranes induced by CAP in normal liver agrees well with the increase of liver protein and supports the assumption that the observed effects are the result of a liver detoxication mechanism much more efficient than in regenerating liver, combined with a much slower rate of mitochondrial formation.

The fact that the protein specific radioactivity of the light mitochondrial fraction is affected by CAP differently from those of the microsomal membrane and ribosomes, both in regenerated and in normal liver, discards any considerable contamination of the outer membrane by fragments of the endoplasmic reticulum. This possibility had been indicated by Allmann, Bachmann, Orme-Johnson, Tan & Green (1968) to explain the discrepancies between their procedure of mitochondrial subfractionation and that of Sottocasa *et al.* (1967a). This also suggests that both membranes are synthesized by separate systems with a different sensitivity towards CAP. The inhibition of the incorporation of amino acids into the proteins of the light mitochondrial fraction conflicts with an extrapolation of the hypothesis put forward for yeast by Clark-Walker & Linnane (1967) assuming that the outer mitochondrial membrane would be synthesized on cytoplasmic ribosomes insensitive to the antibiotic. It appears evident that CAP exerts more complex effects on the protein-synthesizing mechanism of the intact

mammalian cell *in vivo* than those derived from the selective inhibition of 70S ribosomes observed in yeast or in cell-free systems.

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