EFFECTS OF CORTISONE ADMINISTRATION ON RAT LIVER MITOCHONDRIA

Support for the Concept of Mitochondrial Fusion

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ABSTRACT

The present studies investigate the basis for the marked increase in mitochondrial size and approximately reciprocal decrease in mitochondrial number which have been observed in the livers of rats treated with cortisone acetate. Comparisons of the content and specific activity of mitochondrial DNA in the livers of control and cortisone-treated animals prelabeled with radioactive thymidine support the possibility that these changes in mitochondrial size and number are the result of a process of mitochondrial fusion. A consideration of various conditions now known to result in the formation of large mitochondria in other systems suggests that interference with mitochondrial respiration may provide a stimulus for such a process. The biochemical approach described in the present study may prove useful in investigating the origin of large mitochondria in other systems as well.

Effects of the glucocorticoid hormones on a variety of mitochondrial functions have been a subject of interest for many years. While it has long been known that cortisone treatment results in marked changes in a number of mitochondrial biochemical functions (1–3), it has only recently been recognized that these biochemical changes are accompanied by striking morphological alterations. Although it had been observed as early as 1955 that the administration of cortisone to rats appeared to result in both a decrease in number of mitochondria and an increase in mitochondrial size in hepatic parenchymal cells (4), it was not until 1968 that quantitative morphometric studies were performed which conclusively demonstrated that administration of cortisone acetate for a period of 6 days leads to a striking increase in the size of liver mitochondria (3). These latter studies showed that there was a generalized increase in mitochondrial size and that the magnitude of the increase depended upon the portion of the liver lobule examined. Thus the increase in mitochondrial size was shown to range from a 35 per cent increase in average volume for mitochondria in centrilobular cells to a more than 4-fold increase for mitochondria in peripheral and midzonal cells. Subsequent sucrose density gradient studies of *unfixed* rat liver mitochondria have confirmed the fact that cortisone treatment results in a marked increase in mean mitochondrial volume (5).

While the increase in mitochondrial size during cortisone treatment was a striking observation in itself, perhaps the most interesting feature of the electron microscope studies was the demonstration that the increase in mean mitochondrial volume was accompanied by a decrease in number of mitochondria which, in all portions of the liver lobule, was very nearly the exact reciprocal of the increase in mitochondrial size (3). Table I presents a summary of some of these earlier data and shows that, despite marked changes in mitochondrial size and number, the total mitochondrial volume per cell and the percent of the total cytoplasmic volume

Table I

*Mitochondrial Characteristics in Control and Cortisone-Treated Rats**

	Portion of liver lobule		
	Central	Mid-zonal	Peripheral
Volume of average mitochondrion (μ^3)			
Control	0.50	0.70	0.85
Treated	0.67	3.29	3.48
Number of mitochondria per cell			
Control	1530	1480	1160
Treated	1320	430	370
Total mitochondrial volume per cell (μ^3)			
Control	765	1046	995
Treated	886	1415	1280
% cytoplasmic volume occupied by mitochondria			
Control	15.0	20.5	19.5
Treated	15.3	24.4	22.1

* Adapted from Kimberg et al. (3)

occupied by mitochondria remain relatively unaltered in all portions of the liver lobule during cortisone treatment. In addition to the conservation of total mitochondrial volume, the calculated amount of cristal surface has been found to be unchanged (3), and the yield of mitochondrial protein per gram of liver has subsequently been shown to be unaffected by cortisone treatment (see below).

The observation of reciprocal changes in mitochondrial size and number is open to a number of different interpretations, and, among these, two extreme models present themselves as possibilities: reciprocal changes in mitochondrial size and number could reflect a loss of certain mitochondria accompanied by growth of others; or, instead, such changes might be due to a process of mitochondrial fusion. There is now good evidence that mitochondria are capable of exchanging genetic information (6), and while the results of purely morphologic studies have suggested that mitochondria under certain conditions may actually be capable of undergoing fusion (see, *e.g.*, reference 7), the latter possibility has not as yet been conclusively demonstrated (see Discussion).

The central question to be resolved in attempting to distinguish between mitochondrial fusion and mitochondrial "dropout" followed by resynthesis is whether or not the experimentally induced changes in mitochondrial size and number are accompanied by conservation of preexisting mitochondrial material. By the use of isotopic techniques the present study examines the fate of a number of mitochondrial components in the livers of cortisone-treated rats. The results to be presented complement the previous morphologic observations and provide additional evidence in support of the concept of cortisone-induced mitochondrial fusion.

MATERIALS AND METHODS

Materials

Studies were performed on normal male Sherman rats of the weights indicated below and maintained on food and water ad lib. Ethidium bromide was obtained from the Gallard-Schlesinger Chemical Mfg. Corp., Long Island, N. Y. Cesium chloride (optical grade) was obtained from The Harshaw Chemical Co., division of Kewanee Oil Company, Cleveland, Ohio. Deoxyribonuclease, type I, was purchased from the Worthington Biochemical Corp., Freehold, N. J. L-leucine-^{14}C (uniformly labeled, 250 mCi/mmole) and thymidine-^{3}H (methyl-labeled, 6.7 mCi/μmole) were purchased from the New England Nuclear Corp., Boston, Mass. Cortisone acetate was obtained from Merck, Sharp & Dohme, West Point, Pa.

Determination of Mitochondrial Protein Turnover

After an overnight fast, rats weighing approximately 150 g were injected intraperitoneally with 0.5 ml of a solution containing 30 μCi of leucine-^{14}C in isotonic saline. 24 hr after injection, four animals ("zero-time controls") were stunned by a blow to the head and exsanguinated. The livers were rapidly excised and rinsed in chilled 0.25 M sucrose, and mitochondria from individual livers were prepared by the method of Schneider and Hogeboom (8) as modified by Weinbach (9) and washed a total of five times in 0.25 M sucrose. The washed mitochondria were extracted by the procedures described by Beattie et al. (10) in order to remove fractions referred to by these authors as "water-soluble proteins", "cytochrome *c*", "contractile protein", "structural protein", and "other cytochromes". Because of the heterogeneity of the protein fractions obtained by these procedures, we have referred to these preparations as fractions I through V, respectively. The proteins of a sample of the final mitochondrial suspension and of each of the fractions were precipitated with trichloroacetic acid at a final concentration of 5% and extensively washed by the method described by Siekevitz (11). The proteins were dissolved in 0.4 M NaOH, and samples were removed for determination of protein and radioactivity. Protein was measured by a modification ot the Folin-Ciocalteu method (12). Radioactivity was subsequently determined in Bray's solution (13) by means of a Packard liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Counting efficiency was 80%.

24 hr after the injection of leucine-^{14}C the remaining rats were randomly divided into two groups, and on that day and each day thereafter, including the day before sacrifice, each animal received a subcutaneous injection of either 0.2 ml of cortisone acetate suspension (25 mg per ml) or 0.2 ml of 0.15 M NaCl ("control"). After 3, 7, 11, 14, and 19 days of treatment, four control and four cortisone-treated rats were sacrificed and the mitochondria were prepared and fractionated as described for the animals killed at zero-time.

Determination of Content and Specific Activity of Mitochondrial DNA

In four separate experiments, rats weighing approximately 120 g were injected intraperitoneally, at 9:00 a.m. 12 days before sacrifice, with 0.2 ml of an aqueous solution containing 200 μCi of thymidine-^{3}H. The injection of labeled precursor was repeated at 5:00 p.m. and again at 9:00 a.m. on the following day (*i.e.*, each animal received a total of 600 μCi of thymidine-^{3}H). 6 days before sacrifice, rats were randomly divided into two groups of eight animals each (16 animals for each experiment). On that day and on each day thereafter, including the day before sacrifice, each animal received a subcutaneous injection of either 0.2 ml of cortisone acetate suspension (25 mg per ml) or 0.2 ml of 0.15 M NaCl (control).

After this 6-day course of treatment the animals were stunned by a blow to the head and exsanguinated. The livers were rapidly excised and rinsed in chilled buffer, and mitochondria were isolated as described in detail elsewhere (14). DNA was extracted and determined as previously reported (14, 15), and protein was determined as described above. The absence of significant amounts of nuclear DNA in DNA preparations from DNAase-treated mitochondria was demonstrated in appropriate control studies by equilibrium sedimentation of the DNA in cesium chloride gradients in the presence of ethidium bromide (14, 16–18). This method is capable of cleanly resolving intact circular mitochondrial DNA from nicked mitochondrial DNA and from nuclear DNA as has been shown in numerous previous studies employing combined ultracentrifugation and electron microscope techniques (16, 17, 19, 20). Radioactivity in mitochondrial DNA was measured in acid hydrolysates of DNAase-treated mitochondria as previously reported (14). Counting efficiency was 20%.

RESULTS

In order to investigate the basis for the previously observed marked changes in mitochondrial size and number which occur during cortisone administration, the effects of cortisone treatment on the specific activity and yield of prelabeled mitochondrial proteins and mitochondrial DNA were examined. Since calculations based on the data of Kimberg et al. (3) show that cortisone treatment for a period of 6 days results in a 58 per cent decrease in the average number of mitochondria per liver cell, such an approach might permit one to distinguish between two extreme models which can be proposed to account for these changes: (*a*) administration of cortisone results in a fusion of mitochondria with conservation of preexisting mitochondrial components; or (*b*) administration of cortisone results in a loss of 58 per cent, or approximately three fifths of the preexisting mitochondria and a concomitant growth of remaining mitochondria. In contrast to the former model the latter would predict that mitochondria from the livers of rats previously labeled in vivo with radioactive precursor should show a markedly accelerated disappearance of label during cortisone treatment.

In a preliminary series of experiments rats were

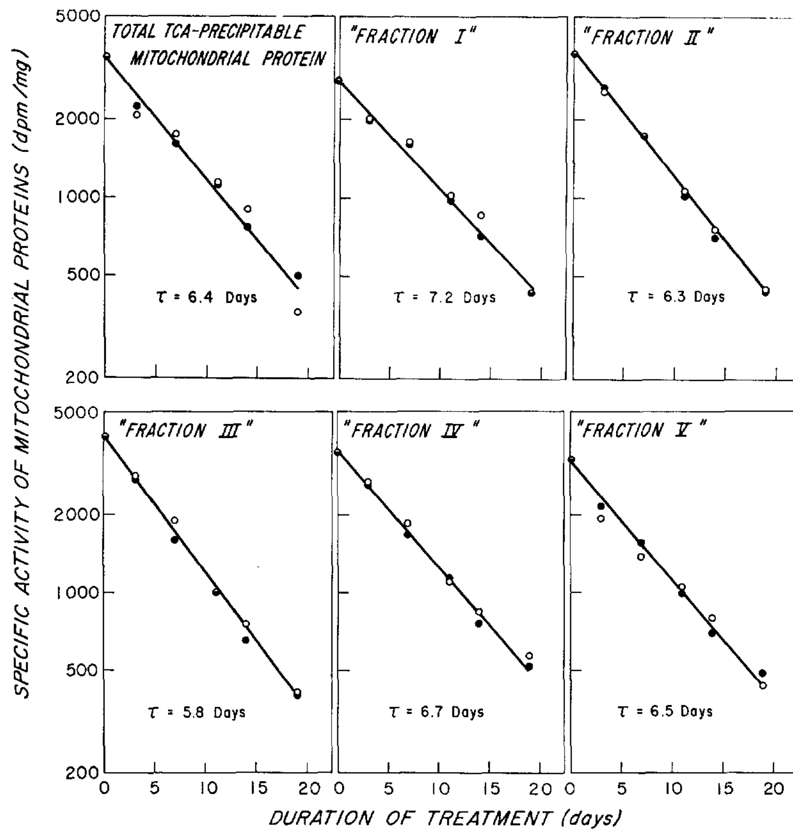

Figure 1 Decline in specific activity of various rat liver mitochondrial protein fractions from control animals (solid circles) and cortisone-treated animals (open circles) prelabeled with leucine-^{14}C. Note that the decline is semilogarithmic and unaffected by cortisone treatment. The protein fractions were prepared as described by Beattie et al. (10) and are defined under Materials and Methods.

prelabeled with radioactive leucine, and the turnover of various mitochondrial protein fractions was determined as described under Materials and Methods. The results are shown in Fig. 1. The observed rate of protein turnover in control animals was found to be similar to that reported for normal animals in numerous previous studies (see, *e.g.*, references 21 and 22), and, more important, the rate of loss of label from all mitochondrial fractions can be seen to be identical in control and cortisone-treated animals.

The observation that the normal rate of turnover of mitochondrial protein is unperturbed in the cortisone-treated animals provides suggestive evidence that the administration of hormone does not result in a superimposed loss of three fifths of the preexisting mitochondrial material within 6 days. The possibility, however, that such a loss does occur and that the apparent lack of accelerated turnover in the treated animals is due to extensive reutilization of radioactive amino acid in a concomitant growth of the remaining two fifths made it desirable to apply another approach.

The approach that was chosen was to determine the effects of cortisone treatment on the yield and specific activity of mitochondrial DNA which had been prelabeled with radioactive thymidine before the onset of treatment. On the basis of the recent demonstration by Gross et al. that liver mitochondrial DNA has a half-life of 9.4 days (23), one can calculate that over the course of 6 days (the duration of treatment in the present series of experiments) one would expect, on the basis of spontaneous turnover, a maximal fall in the specific activity of mitochondrial DNA of no more than 35 per cent in the control group. If in the cortisone-treated animals there is a superimposed loss of three fifths of the prelabeled mitochondrial DNA,

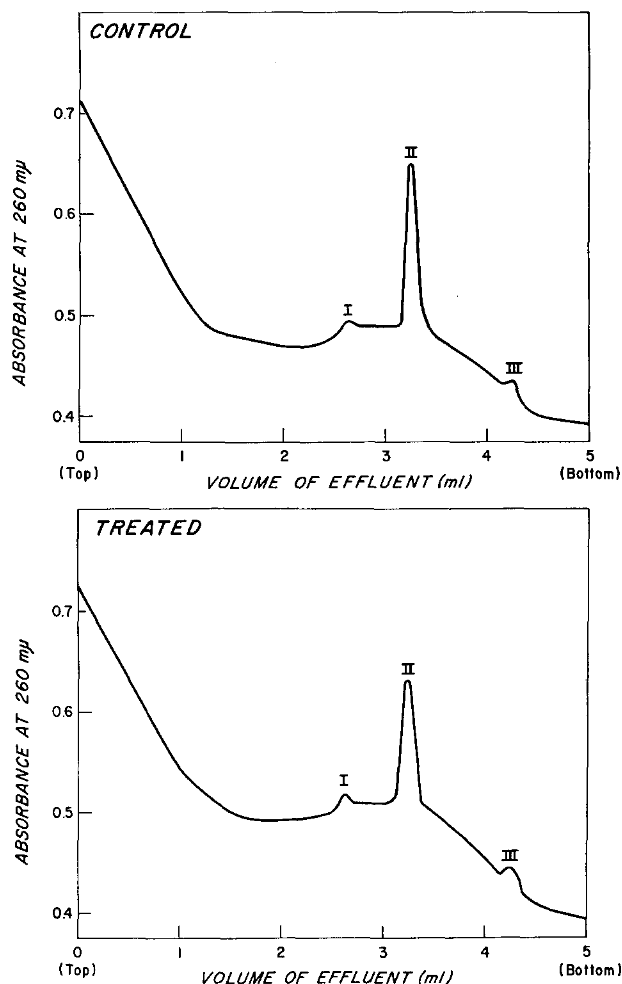

FIGURE 2 Absorbance (2 mm light path) at 260 mμ of the effluent from a CsCl-ethidium bromide gradient containing DNA extracted from washed, DNAase-treated mitochondria from livers of control and cortisone-treated animals. The predominant band (peak II) represents intact circular mitochondrial DNA and consistently contains over 85% of the total DNA; DNA banding at a lower density (peak I) consists of nicked mitochondrial DNA and/or nuclear DNA (17). The falling "baseline" absorbance is due to the decreasing concentration of ethidium bromide in the gradient; peak III represents turbidity due to precipitated glycogen. The isopyknic centrifugation characteristics of mitochondrial DNA are seen to be unaltered by cortisone treatment.

the differences in the amount of radioactivity in mitochondrial DNA in control and cortisone-treated animals should be readily apparent by the end of the 6-day period of treatment

Before attempting such experiments it was essential to establish both the purity and the reproducibility of the yield, per milligram of mitochondrial protein[1], of mitochondrial DNA. Fig. 2 shows the results of isopyknic centrifugation of representative samples of DNA isolated from mitochondria from control and hormone-treated animals in cesium

[1] While the yield of protein from mitochondria purified as described above shows a certain variability even in control animals, numerous experiments have demonstrated that the yield is not significantly affected by 6 days of cortisone treatment. Because it is not possible by techniques currently available to obtain purified rat liver mitochondria in quantitative yield, results are expressed per milligram of mitochondrial protein.

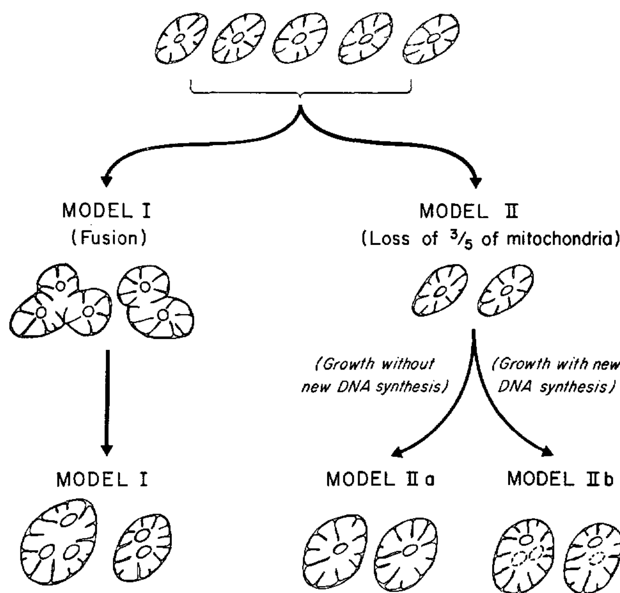

FIGURE 3 Three models to account for the observed decrease in mitochondrial number and reciprocal increase in mitochondrial size in the livers of cortisone-treated rats. Small circles within the mitochondria represent mitochondrial DNA: solid circles, preexisting (radioactive) DNA; dotted circles, newly synthesized (nonradioactive) DNA.

chloride density gradients in the presence of ethidium bromide. The DNA preparations from control and cortisone-treated animals show identical sedimentation patterns. The denser of the two DNA peaks (peak II), accounting in our preparations for at least 85% of the total DNA, has been shown in numerous previous studies to represent intact (circular) mitochondrial DNA (16, 17, 19, 20). The yield of mitochondrial DNA is reproducible from experiment to experiment and is similar in both groups of animals (see below).

The following series of experiments was undertaken to determine the effects of cortisone treatment upon the specific activity and yield of mitochondrial DNA from animals prelabeled with radioactive thymidine, and hence to distinguish between several models which might be proposed to explain the previously observed reciprocal changes in mitochondrial size and number. A number of such models lead to markedly different predictions for the effects of cortisone treatment on the specific activity and yield of mitochondrial DNA and hence can be tested by means of such an experiment. The first model, in which there is fusion of mitochondria to form two fifths of the initial number of preexisting units, will be called "model I"; the second, in which there is a loss of approximately three fifths of the mitochondrial units during the course of cortisone treatment and concomitant growth of the remaining two fifths, will be designated as "model II". Model II, in turn, can be considered to have two subcategories: model IIa consisting of growth of the remaining units without new mitochondrial DNA synthesis; and model IIb consisting of growth accompanied by a proportional synthesis of new mitochondrial DNA. A schematic summary of these models is presented in Fig. 3.

Table II shows the data obtained in four experiments in which rats were treated with either saline or cortisone acetate for 6 days after receiving radioactive thymidine. In each case cortisone treatment is seen to have little effect on the yield of mitochondrial protein, the amount of DNA present per milligram of mitochondrial protein, or the specific activity of the isolated mitochondrial DNA. In order to compare the results with the specific models discussed above, Table III presents a summary of the predictions on the basis of each of the models shown in Fig. 3 and compares the experimental results for control and cortisone-treated animals. The experimentally observed ratios in each case are seen to be close to unity and are thus most consistent with the predictions of model I and with the conclusion that fusion has taken place.

TABLE II

Effects of Cortisone Treatment on Yield and Specific Activity of Mitochondrial DNA Prelabeled with Radioactive Thymidine

	Experiment No.				
	1	2	3	4	Mean ± 1 SE
μg mitochondrial DNA per mg mitochondrial protein					
Control	0.162	0.241	0.145	0.230	0.195 ± 0.028
Treated	0.183	0.219	0.175	0.303	0.220 ± 0.034
specific activity of mitochondrial DNA (dpm per μg mitochondrial DNA)					
Control	333	434	576	510	463 ± 60
Treated	364	513	577	516	493 ± 52
mg mitochondrial protein per g liver					
Control	6.49	6.40	6.00	4.51	5.85 ± 0.46
Treated	5.15	6.35	6.50	5.44	5.86 ± 0.38

TABLE III

Comparison of Experimental Results in Control and Cortisone-Treated Animals with Predictions Based on Different Models

	Ratio to control			
	Predicted (Model I)	Predicted (Model IIa)	Predicted (Model IIb)	Observed*
DNA per mg mitochondrial protein	1.00	0.42‡	1.00	1.14 ± 0.09
Specific activity of mitochondrial DNA	1.00	1.00	0.42‡	1.07 ± 0.04
mg mitochondrial protein per g liver	1.00	1.00	1.00	1.02 ± 0.09

* Mean ± SE (four experiments).

‡ Cortisone treatment for a period of 6 days results in a 58% decrease in the average number of mitochondria per liver cell (see text).

DISCUSSION

The occurrence of large mitochondrial forms has been observed under a variety of different conditions in the past, and from time to time it has been suggested that fusion may play a role in their formation. Nevertheless the observations to date have not been conclusive; not only are fusing forms difficult to identify with certainty, but it might be argued that if the fusion event is rapid, one could not hope to find many such forms in a given number of micrographs. Similarly, the absence of an increased number of degenerating mitochondrial forms is weak and circumstantial evidence at best that the decrease in mitochondrial number which occurs during cortisone treatment is due to fusion rather than to loss of preexisting mitochondria, and difficulties inherent in the interpretation of other purely morphologic evidence have made it desirable to investigate the possibility by other methods as well. The present study was made possible by the fact that while Gross et al. have recently shown that mitochondrial DNA undergoes turnover (23), it does so with a half-life which is sufficiently long with respect to the time required for the cortisone-induced morphologic changes to become established (3) that an investigation of the possibility of mitochondrial fusion by a biochemical approach appeared feasible. The results presented in this study describe such an approach and support the conclusion that during the course of cortisone treatment mitochondrial fusion does indeed take place

(model I). Suggestive as they are, however, it should be borne in mind that the results presented here do not constitute a definitive proof: although improbable, one cannot exclude the possibility that the thymidine-^{3}H present in a degenerating three fifths of the mitochondria is quantitatively reutilized for new DNA synthesis in the remaining two fifths (model IIb). Furthermore, the interpretation of the results is based upon the assumption that the composition of the purified mitochondrial samples studied was representative of the total mitochondrial population; techniques which are currently available do not permit complete recovery of isolated mitochondria in purified form from homogenates of liver.

Some of the most striking biochemical alterations in mitochondrial function which appear during the course of cortisone administration are multiple defects in the respiratory chain (1, 3), and it is tempting to speculate that the hormone-induced inhibition of mitochondrial respiration may in some way serve as a stimulus for mitochondrial fusion. This idea would seem to be particularly attractive in view of the observations that a number of other conditions which may interfere with mitochondrial respiration are known to result in the formation of large mitochondria. Thus, iron- (24, 25), copper- (24), and manganese- (26) deprivation, the administration of the copper-chelating agent cuprizone (27), and, perhaps most dramatically, the addition of antimycin A to cultures of a bleached strain of *Euglena gracilis* (7) have all been shown to be associated with the formation of large mitochondria. The possibility that mitochondrial fusion may play a role in some of these other systems if of interest and is the subject of studies currently in progress.

This work was supported in part by United States Public Health Service Grants AM-13696, AM-05114, CA-10736, GM-15022, HD-05506, and AM-05397, and by an institutional grant from the American Cancer Society (IN-4L). Dr. Kimberg is the recipient of a Research Career Development Award, AM-19377.

Received for publication 15 May 1972, and in revised form 13 July 1972.

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