

## EFFECTS OF CORTISONE ON RAT LIVER MITOCHONDRIA\*

BY CHARLES UPTON LOWE, M.D., DELORIS MACKINNEY, AND  
DALJIT SARKARIA, PH.D.

(From the Staller Research Laboratories of the Children's Hospital and the Department  
of Pediatrics, University of Buffalo School of Medicine, Buffalo)

PLATES 64 AND 65

(Received for publication, December 23, 1954)

Biochemical studies indicate that a substantial decrease in mitochondrial ribonucleic acid (RNA) of rat liver cells occurs following parenteral administration of large doses of cortisone acetate (1). Reports have appeared indicating that carcinogens can also affect mitochondrial RNA content as well as their number (2, 3), while virus invasion of a cell alters mitochondrial morphology (4). Mitochondria appear to be the locus of action of both the Krebs tricarboxylic acid enzyme system and the cytochrome system. Alterations, therefore, in their numbers might have important implications with respect to cell physiology as influenced by cortisone.

From 10 to 20 per cent of the total RNA of the cell (5) is regularly associated with hepatic mitochondria. Basophilic stains such as pyronine (6), toluidine blue (7), and gallocyanin chrome alum (8) appear to have an affinity for RNA, and in the normal liver cells they can be used to demonstrate considerable amounts of this material. When, however, they are used to stain liver cells from animals subjected to chemical injury (7), starvation (9), or cortisone treatment (1, 10-13) one observes a marked depletion in basophilic material.

To determine whether the fall in mitochondrial RNA following cortisone administration represents a decrease in mitochondrial numbers or merely a loss of RNA, the studies described below were undertaken.

A comparison of mitochondrial numbers was made between normal rat liver and liver from rats that had received cortisone. Three experimental approaches were used:

1. Small blocks of rat liver were fixed in an osmic acid-containing mixture, stained appropriately, and examined for mitochondria.
2. Fresh, unstained, whole liver cells were examined for mitochondria with the phase microscope.
3. Mitochondrial and nuclear counts were performed on fresh whole liver homogenates.

\* Supported by grants from the United States Atomic Energy Commission, the National Institutes of Health, and the American Cancer Society.

### Materials and Methods

The methods used are presented in considerable detail since the published information on counting procedures is frequently confusing and often difficult to follow.

In all experiments male rats of the Wistar strain weighing between 100 to 200 gm. were used. They were maintained on a Lablox R diet<sup>1</sup> and fed *ad libitum*.

The animals were divided into two groups: (1) normal control and (2) cortisone-injected. These received daily 25 mg. of cortisone acetate<sup>2</sup> by intramuscular injections for a period of 5 days. Both groups of rats were starved for 24 hours and then, while under light anesthesia, exsanguinated by severing the hepatic vein.

1. *Procedure for Selective Staining of Mitochondria.*—At the time of sacrifice  $2 \times 2 \times 2$  mm. blocks of liver were obtained and fixed immediately in Flemming's fluid<sup>3</sup> and stained with iron hematoxylin (14). Under these conditions mitochondria appear as solid gray-blue spheres.

2. *Examination of Fresh Unstained Whole Liver Cells with Phase Microscope.*—Fresh whole liver parenchymal cells from both cortisone-injected and control rats were teased apart in an iced solution of 0.88 M sucrose. They were immediately examined with a phase microscope.<sup>4</sup>

3. *Procedure for Determining the Number of Mitochondria per Nucleus.*—The method used is similar in most details to that of Shelton, Schneider, and Striebich (15). While the rat was under light ether anesthesia the liver was removed and forced through an ice cold stainless steel meat press with holes of 0.1 cm. The liver pulp was then divided into two portions which were separately weighed. With the aid of an iced Potter-Elvehjem grinder equipped with a teflon plug,<sup>5</sup> the first portion was homogenized and then diluted in an iced, 0.25 M sucrose solution. The second portion was homogenized in the same way in an iced, slightly alkaline 0.85 per cent sodium chloride solution. The final volume of the homogenates was so adjusted that in the sucrose solution the liver was in a 10 per cent suspension, whereas in the saline solution it was in a 20 per cent suspension (*w/v*). Mitochondria were counted in the sucrose solution and nuclei in the saline solution. All operations were conducted as near 4°C. as possible with the exception of the actual counting. All counting was performed in a Petroff-Hausser bacteria-counting chamber with a phase contrast microscope ( $\times 10$  ocular,  $\times 97$  oil immersion, dark-light contrast objective, with Kohler illumination).

### Counting of Mitochondria

4 cc. of the sucrose suspension of normal livers, or 5 cc. of suspension of livers from cortisone-injected animals were withdrawn in a volumetric pipette. These amounts were diluted with 0.25 M sucrose to 10 ml. in a volumetric flask. From these dilutions, a NBS (National Bureau of Standards) red cell diluting pipette was filled to the 1.0 mark. Sucrose, 0.25 M, was drawn to the 101 mark resulting in a 1:250 dilution for normal livers and a 1:200 for livers from cortisone-injected animals.

Three pipettes were filled and stored at 4°C. to be examined within 1 hour and each pipette was shaken on a Burton shaker for 5 minutes immediately before use. One count was made from each pipette. Only the center ruling was utilized in the counting chamber, which had an improved Neubauer grid. Eighty of the smallest squares in five groups of sixteen were counted. Five groups of sixteen small squares represent a volume of 0.004 mm.<sup>3</sup> For increased accuracy, and following the indications of Sanford *et al.* (16), a total of at least 370 mitochondria was

<sup>1</sup> Allied Mills.

<sup>2</sup> Kindly supplied by Merck and Co., Inc.

<sup>3</sup> 15 parts 1 per cent chromic acid; 4 parts 2 per cent osmic acid.

<sup>4</sup> American Optical Company, dark-light contrast,  $\times 97$  objective.

<sup>5</sup> Arthur H. Thomas Co., Philadelphia.

counted for each count. The total number of mitochondria was averaged for eighty small squares and by simple arithmetic, this was converted to mitochondria per gram of whole liver.

### *Recognition of Mitochondria*

Mitochondria usually appeared as individual gray spheres from 0.5 to 2 microns in diameter. Their borders were intact and slightly darker than the interior. Usually the border appeared wider on one side forming a crescent similar to that described by Harman (17). The smallest forms seen, which appeared as solid black spheres with smooth limiting membranes, had to be distinguished from similar forms having an asymmetrical outline with a suggestion of irregularity of the limiting membrane. Experiments were undertaken on the assumption that mitochondria swell in hypotonic solutions and in the presence of detergents (17). The two forms mentioned above were observed in 0.25 M sucrose in the counting chamber at a time, when either distilled water or a detergent<sup>6</sup> had been added. The small symmetrical particles remained intact; all other forms swelled and became unrecognizable. Consequently, those particles unaffected by the detergent or distilled water were not counted as mitochondria.

It was essential, in order to fill the chamber properly, to have it absolutely clean. Thus, immediately following each count, the chamber and coverslip were washed in warm detergent, warm tap water, and cold distilled water. Mitochondria in a properly filled chamber exhibited Brownian movement. Any other movement, such as streaming, made counting impossible and such improperly filled chambers were discarded. With passage of time there appeared to be a decrease in the number of mitochondria in a given suspension. Therefore, all counts were completed within 1 hour of preparation.

### *Counting of Nuclei*

From the alkaline saline suspension a sample was withdrawn with a NBS white cell diluting pipette to the 1 mark. A dilution was made to the 11 mark with a solution of 3 per cent methyl green in iced alkaline saline resulting in a 1:10 dilution. After a rubber band had been slipped over the ends of the pipette, it was shaken for a minimum of 15 minutes on a Burton pipette shaker before being examined.

It was found to be exceedingly important, before filling the counting chamber and after the first few drops had been discarded from the pipette, to agitate the pipette again by hand to disperse any large aggregates of nuclei.

At least three counts from separate pipettes were made to insure greater accuracy. Counting was performed in a manner similar to that used for counting mitochondria. However, instead of eighty small squares, 400 were counted having a volume of 0.1 mm.<sup>3</sup> The nuclei per gram of whole liver could then be calculated. Only parenchymal liver cell nuclei (identified by size and morphology) were included in the total count.

From the results of these measurements it was possible to calculate the number of mitochondria per nucleus. However, a more significant observation concerning the action of cortisone on liver cells might be the number of mitochondria per cell. Many liver cells normally have more than one nucleus; therefore, to derive the value for mitochondria per cell it is necessary to apply an appropriate correction.

From each animal studied, a piece of liver was fixed in Lavdowsky's solution and subsequently stained with hematoxylin and erythrosin. These sections were closely examined for multiple nuclei and the counts obtained were handled in the way suggested by St. Aubin and Bucher (18). Since there was no difference between the control and cortisone-injected animals, with respect to the number of binucleate cells, the relative values of mitochondria per nucleus for both groups served equally as well as mitochondria per cell to demonstrate any change in the numbers of mitochondria.

<sup>6</sup> Dural, Harshaw Scientific Company, Cleveland.

## RESULTS

1. *Selective Staining of Mitochondria.*—Comparison between the livers from normal and from cortisone-treated animals indicates a striking reduction in the number of mitochondria as revealed by iron hematoxylin staining after hormone administration. Those mitochondria remaining appear larger than the average of the normal and they stain more deeply at their periphery, giving an empty appearance. These findings are illustrated in Figs. 1 *a* and *b*.

2. *Examination of Fresh Unstained Liver Cells with Phase Microscopy.*—The same difference, which could be seen in the fixed tissue, was evident in the fresh preparations. There appeared to be a decrease in the number of mitochondria in the liver cells of cortisone-treated animals and in association with the decrease in number there was a swelling of most of the mitochondria. Photomicrographs of such preparations are seen in Fig. 2.

TABLE I  
*Effect of Cortisone on Mitochondrial Numbers of Rat Liver Cells\**

	Mitochondria $\times 10^6$ per gm. wet liver	Nuclei $\times 10^6$ per gm. wet liver	Mitochondria per nucleus
Controls (20)	105 $\pm$ 1.06	160 $\pm$ 22	834 $\pm$ 108
Cortisone (17)	41.8 $\pm$ 3.9	159 $\pm$ 12	278 $\pm$ 25.7

\* Average and S.E. of mean. Numbers in parentheses indicate number of experiments.

† Probability determined by *t* test.

3. *Mitochondria and Nuclear Counts on Fresh Whole Liver Homogenates.*—The results of these experiments are tabulated in Table I.

The average number of mitochondria per cell in the cortisone-injected rats was 40 per cent that of the normal.

## DISCUSSION

The figures for mitochondria per gram of liver for the controls are in favorable agreement with those reported by Striebich, Shelton, and Schneider ( $118 \times 10^6$ ) (19). The nuclear counts per gram of liver are similar to those of Stevens, Daoust, and Leblond ( $133.8 \times 10^6$ ) (20), Price, Miller, Miller, and Weber ( $137 \times 10^6$ ) (2), and Brues, Drury, and Brues ( $143 \times 10^6$ ) (21). It seems doubtful, therefore, that any gross error in counting technique could explain the results reported.

Of the three types of experiments performed, each appears to support the conclusion that cortisone decreases the number of mitochondria per liver cell. The most conclusive of the three is the experiment in which mitochondria were enumerated in a counting chamber and related to the number of nuclei

present. However, the histological observations are none the less important and are necessary for adequate interpretation and support of the counting data. For instance, it may be argued that the swollen mitochondria observed in the liver cells of cortisone-treated rats (Figs. 1*b* and 2*b*) might be more fragile than those found in normal animals. If the swollen forms be more fragile, it is conceivable that they disintegrate during the short interval that elapses between preparation and enumeration. This view may derive some support from the fact that during the counting procedure no obvious difference in size is observed between mitochondria isolated from normal liver and mitochondria obtained from the liver of cortisone-treated animals. The explanation, however, seems unlikely, for in the sections of liver fixed in osmic acid, seconds after removal from the animal (Fig. 1) and stained with iron hematoxylin, one observes decreased numbers of mitochondria. In these sections homogenization and manipulation prior to counting could not have influenced mitochondrial numbers. In the mitochondrial preparations concerned it is probable that other factors are responsible for the lack of difference in size observed after isolation.

Certain enzymes appear to function exclusively in relation to mitochondria (22, 23). Schneider *et al.* (3) have demonstrated fluctuation in the activity of mitochondrial enzymes with change in mitochondrial number in rat liver cells; there was no concomitant alteration in the RNA of the whole cell. One might anticipate, following cortisone administration, a decrease in activity of those enzymes related to mitochondrial structure to the same degree that the mitochondrial numbers decreased. This would be attractive reasoning for it might help to explain some of the metabolic consequences upon administration of cortisone to animals and man. Unfortunately the data of Lowe and Lehninger (24) do not support this hypothesis for they were unable to find any significant difference between livers of normal and livers of cortisone-treated rats with respect to succinoxidase activity and oxidative phosphorylation. This by no means, however, eliminates the possibility that one of the ways by which cortisone influences metabolism is by affecting the mitochondria or the enzyme systems with which they are associated.

#### SUMMARY AND CONCLUSIONS

Experiments were designed to determine the effects of parenteral administration of cortisone on mitochondrial numbers of rat liver cells. Observations were made in three ways: (1) after osmic acid fixation and appropriate stain, (2) by phase microscopy on intact cells, (3) by actual enumeration of mitochondria and nuclei to provide data on mitochondria per cell. The results of the experiments indicate that the mitochondria per cell are significantly reduced under the conditions used. Each experimental approach provided similar data, and each tended to support the results obtained from the others.

## BIBLIOGRAPHY

1. Lowe, C. U., and Williams, W. L., Effect of cortisone administration on intracellular composition of rat liver, *Proc. Soc. Exp. Biol. and Med.*, 1953, **84**, 70.
2. Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., Studies on the intracellular composition of livers from rats fed various aminoazo dyes. II. 3-Methyl, 2-methyl, and 2-methyl-4 dimethylaminoazobenzene, and 4'-fluoro-4-dimethylaminoazobenzene, *Cancer Research*, 1950, **10**, 18.
3. Schneider, W. C., Hogeboom, G. H., Shelton, E., and Striebich, M. J., Enzymatic and chemical studies on the livers and liver mitochondria of rats fed 2-methyl- or 3'-methyl-4-dimethylaminoazobenzene, *Cancer Research*, 1953, **13**, 285.
4. Bang, F., Cellular changes in the chick chorio-allantoic membrane infected with herpes simplex and vaccinia, *Bull. Johns Hopkins Hosp.*, 1950, **87**, 511.
5. Hogeboom, G. H., Schneider, W. C., and Palade, G. E., Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material, *J. Biol. Chem.*, 1948, **172**, 619.
6. Michaelis, L., The nature of the interaction of nucleic acids and nuclei with basic dyestuffs, *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12**, 131.
7. Williams, W. L., Cytoplasmic changes in hepatic parenchyma of mice during starvation and carbon tetrachloride induced injury, *Anat. Rec.*, 1951, **111**, 629.
8. Lagerstedt, S., Cytological studies on the protein metabolism of the liver in the rat, *Acta Anat.*, suppl. IX, 1949.
9. Davidson, J. N., Some factors influencing the nucleic acid content of cells and tissues, *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12**, 50.
10. Williams, W. L., Lowe, C. U., and Thomas, L., The effects of cortisone upon the parenchymal cells of the rabbit liver, *Anat. Rec.*, 1953, **115**, 247.
11. Lowe, C. U., Williams, W. L., and Thomas, L., Effect of cortisone upon nucleic acid composition of rabbit liver, *Proc. Soc. Exp. Biol. and Med.*, 1951, **78**, 818.
12. Timiras, P. S., and Koch, P., Morphological and chemical changes elicited in the liver of the rabbit by cortisone and DOCA, *Anat. Rec.*, 1952, **113**, 349.
13. Gros, F., Bonfils, S., and Macheboeuf, M., Influence de la cortisone sur la teneur en acides nucleiques de divers tissus, *Compt. rend. Acad. sc.*, 1951, **233**, 990.
14. Lillie, R. D., *Histopathologic Technique*, Philadelphia, The Blakiston Company, 1948.
15. Shelton, E., Schneider, W. C., and Striebich, M. J., A method for counting mitochondria in tissue homogenates, *Exp. Cell Research*, 1953, **4**, 32.
16. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, I. E., The measurement of proliferation in tissue cultures by enumeration of cell nuclei, *J. Nat. Cancer Inst.*, 1951, **11**, 773.
17. Harman, J. W., Studies on mitochondria: II. The structure of mitochondria in relation to enzymatic activity, *Exp. Cell Research*, 1950, **1**, 394.
18. St. Aubin, P. M. G., and Bucher, N. L. R., A study of binucleate cell counts in resting and regenerating rat liver employing a mechanical method for the separation of liver cells, *Anat. Rec.*, 1952, **112**, 797.
19. Striebich, M. J., Shelton, E., and Schneider, W. C., Quantitative morphological

- studies on the livers and liver homogenates of rats fed 2-methyl- or 3-methyl-4-dimethylaminoazobenzene, *Cancer Research*, 1953, **13**, 279.
20. Stevens, C. E., Daoust, R., and Leblond, C. R., Rate of synthesis of desoxyribonucleic acid and mitotic rate in liver and intestine, *J. Biol. Chem.*, 1953, **202**, 177.
  21. Brues, A. M., Drury, D. R., and Brues, M., Quantitative study of cell growth in regenerating liver, *Arch. Path.*, 1936, **22**, 658.
  22. Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., The distribution of cytochrome oxidase and succinoxidase in the cytoplasm of the mammalian liver cell, *J. Biol. Chem.*, 1946, **165**, 615.
  23. Hogeboom, G. H., and Schneider, W. C., Intracellular distribution of enzymes. VIII. The distribution of diphosphopyridine nucleotide cytochrome C reductase in normal mouse liver and mouse hepatoma, *J. Nat. Cancer Inst.*, 1950, **10**, 983.
  24. Lowe, C. U., and Lehninger, A. L., Enzymic oxidation and phosphorylation in liver mitochondria lacking polymerized ribonucleic acid, *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 89.

## EXPLANATION OF PLATES

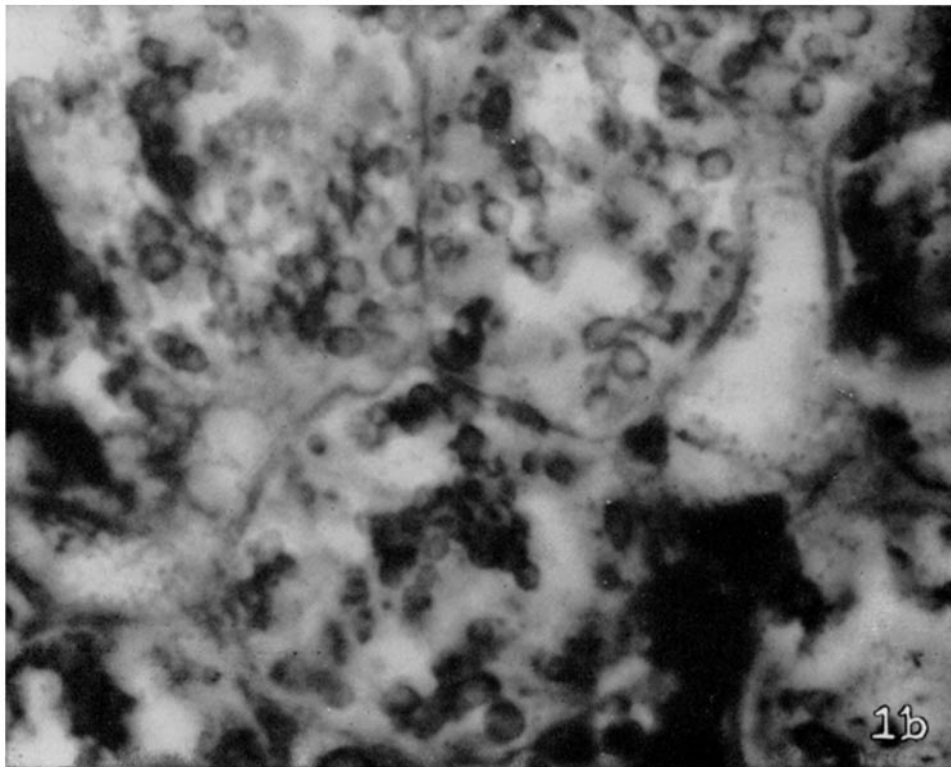
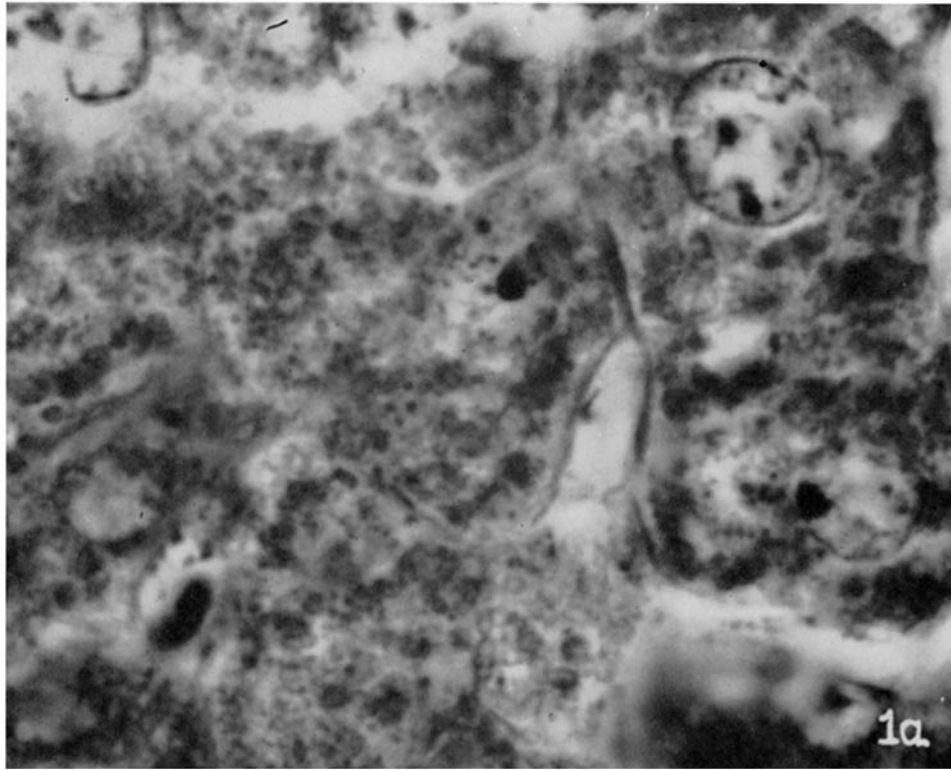
## PLATE 64

FIGS. 1*a* and *b*. Paraffin sections of rat liver fixed in Flemming's fluid without acetic acid and stained with iron hematoxylin.  $\times$  1940.

(*a*) Normal rat. Mitochondria appear as small opaque gray to black spheres and are evenly distributed throughout the cells.

(*b*) Cortisone-injected rat. Mitochondria are enlarged, pale spheres with darkened borders. Their numbers appear reduced.





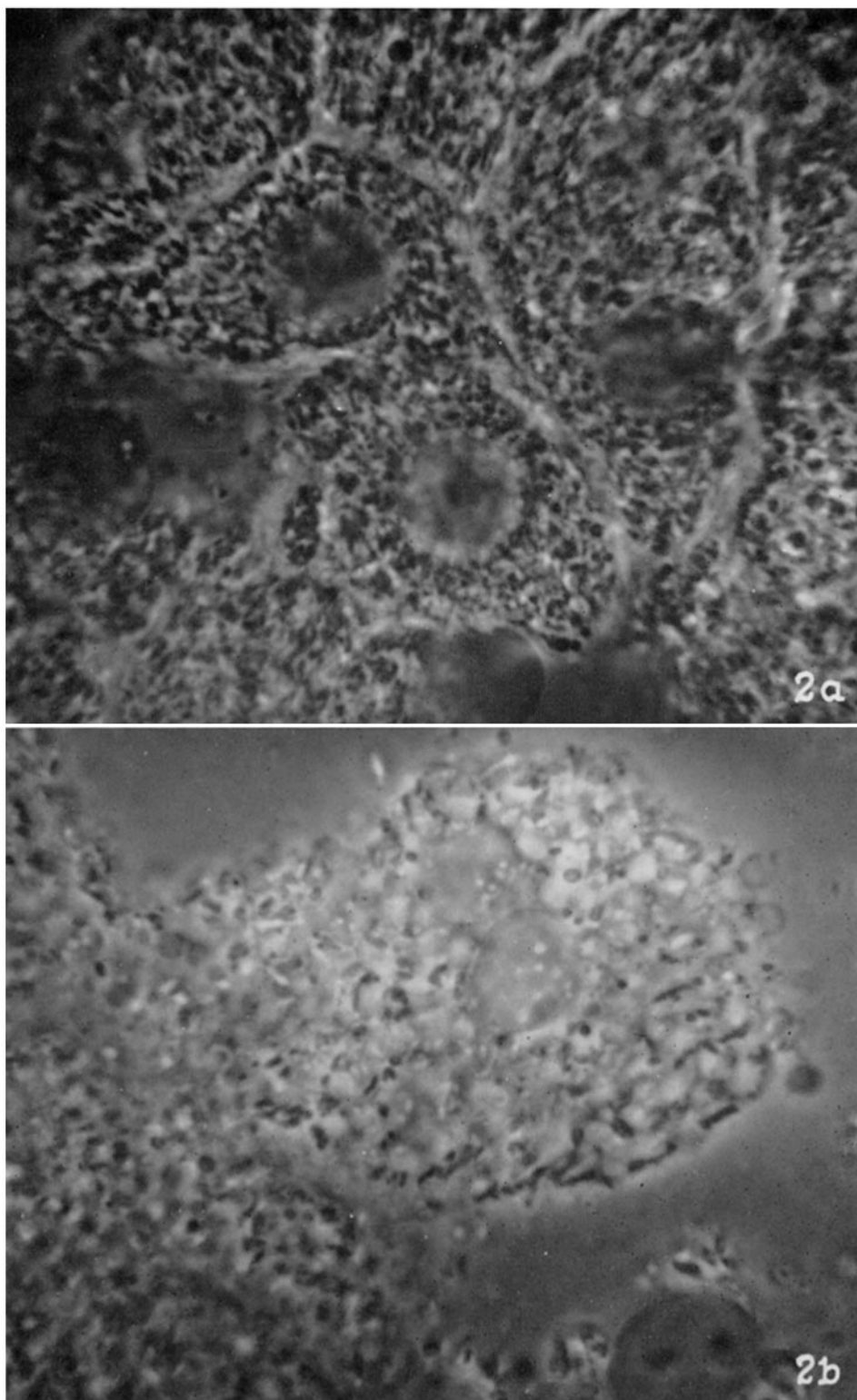
(Lowe *et al.*: Effects of cortisone on rat liver mitochondria)

PLATE 65

FIGS. *2a* and *b*. Unfixed, unstained rat liver in 0.88 M sucrose. Phase microscopy.  
× 1940.

(*a*) Normal rat. Mitochondria appear as small black rods and spheres and are evenly distributed, regular in size, and closely packed in cells.

(*b*) Cortisone-injected rat. Mitochondria are large, pale gray spheres, irregular in size and loosely packed in the cell. A few black rods and spheres are present.



(Lowe *et al.*: Effects of cortisone on rat liver mitochondria)