OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM LIVERS SHOWING CLOUDY SWELLING*,‡

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Changes in the morphology of mitochondria have been observed by several authors during the so-called "cloudy swelling" (1–6) which is a peculiar type of cell degeneration well known to pathologists. In view of the close correlation between the morphology and the enzymatic activity of mitochondria (7–9 and others), some metabolic disturbances occurring during cloudy swelling have been related to the changes in mitochondria (6, 10, 11).

Previous work has shown that phosphorylation of glucose is reduced in diphtheria toxin–treated animals, whose organs show a typical cloudy swelling (10). In these organs, easily hydrolyzable phosphate has been found to be decreased as compared with normal (11). Furthermore, it has been shown that the histological picture of cloudy swelling can be reproduced experimentally by injecting the animals with some compounds which uncouple oxidative phosphorylation, such as 2,4-dinitrophenol (12) and thyroxine (13).

It appeared therefore reasonable to suppose that changes in oxidative phosphorylation could be implicated in the pathogenesis of the aforementioned cellular changes. The present paper concerns the oxidative phosphorylation in mitochondria isolated from livers showing cloudy swelling.

Materials and Methods

Adult albino rats and guinea pigs, bred in the Institute of General Pathology, were used. In order to produce cloudy swelling of the liver, rats were injected intraperitoneally with the smallest dose of *S. typhi murium* toxin that was lethal within 4 days, and guinea pigs were injected subcutaneously with diphtheria toxin (1 M.L.D./250 gm. body weight). *S. typhi murium* toxin was prepared as described previously (6); a commercial preparation of diphtheria toxin was used. The animals were fasted for the 12 hours immediately before killing at 24 hours after the toxin injection.

Preparation of Mitochondria.—All manipulations were performed in a cold room at 0°C. using chilled materials.

The animals were stunned by a blow on the head, bled at the neck, and the liver was quickly excised. A small sample was taken for histological examination and the remainder

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of the liver was immediately cooled in partially frozen 0.25 M sucrose. The tissue was blotted with filter paper and passed through a Fisher mincer. The resulting liver pulp was rapidly weighed and homogenized with 7 volumes of ice cold 0.25 M sucrose containing 0.005 M versene (ethylenediaminetetraacetic acid, adjusted to pH 7.4 with NaOH) in the all-glass apparatus of Potter and Elvehjem (14). The homogenate was centrifuged[1] for 15 minutes at 600 × *g* to remove unbroken liver cells, nuclei, and red blood cells and the sediment discarded. The supernatant was then centrifuged for 20 minutes at 8,500 × *g* to sediment mitochondria; the supernatant and the "fluffy layer" of the deposit were poured off leaving behind the well packed mitochondria. These were then washed twice by resuspending in sucrose-versene (3.0 ml./gm. weight of the original liver pulp) and recentrifuging at 8,500 × *g* for 20 minutes. The final sediment of mitochondria was made up with sucrose-versene to a volume equal to twice the weight of the original liver pulp.

Measurement of Oxidative Phosphorylation.—The principle is to follow the disappearance of inorganic orthophosphate from the reaction medium in the presence of adenosine-5′-phosphate, an excess of glucose, and sufficient hexokinase to obtain the maximum rate of formation of glucose-6-phosphate under the experimental conditions. The reaction was performed in a Warburg manometric apparatus so that the oxygen consumption could be simultaneously measured.

The main compartment of the Warburg flasks contained: 60 μM of potassium phosphate buffer, pH 7.4; 10 μM of versene; 75 μM of KCl; 20 μM of $MgSO_4$; 40 μM of KF; 90 μM of succinate or 30 μM of α-ketoglutarate (both added as sodium salts); 3×10^{-2} μM of cytochrome *c*; 3 μM of adenosine-5′-phosphate (AMP); 78 μM of glucose and, when indicated, 60 μM of potassium malonate, in a total volume of 1.8 ml. The side arm contained 20 mg. of a hexokinase preparation (0.2 ml.). The fluoride was added just before the addition of the mitochondria in order to prevent the precipitation of magnesium fluorophosphate (*cf.* reference 15). 0.2 ml. of 20 per cent (*w/v*) KOH and a roll of filter paper were used in the center well.

The cold preparation of mitochondria (1.0 ml. in 0.25 M sucrose–0.005 M versene) was added to the main compartment of the Warburg flasks. The apparatus was then quickly assembled and shaken in the 25°C. bath for 10 minutes to achieve equilibration. The initial manometer readings were taken, the hexokinase added from the side arm, and the oxygen uptake was measured during the next 20 minutes with air as gas phase. The reaction was then stopped with 0.5 ml. of 85 per cent (*w/v*) trichloroacetic acid, the contents of the flasks made to 5 ml. with water, centrifuged, and filtered. Zero-time controls were performed in which the reaction was stopped at the end of the equilibration period. The trichloroacetic filtrates were kept at 0°C. until the estimation of inorganic orthophosphate. The inorganic orthophosphate which disappeared was calculated from the difference between the inorganic orthophosphate content of various experimental flasks and the corresponding zero-time flasks. From these data and the oxygen uptake during the same time interval, P:O ratios (moles of inorganic orthophosphate which disappear per atom of oxygen consumed) were calculated. The endogenous oxygen uptake of the mitochondrial preparation and the autoxidation of the substrates were negligible.

Dephosphorylating Activities.—The hydrolysis of adenosinetriphosphate (ATP), adenosine-5′-phosphate (AMP), and glucose-6-phosphate (G-6-P) by liver mitochondria has been studied in a reaction mixture as close as possible to that used in phosphorylation experiments. The mitochondria (1.0 ml. in 0.25 M sucrose–0.005 M versene) were added to tubes containing: 60 μM of potassium phosphate buffer, pH 7.4; 10 μM of versene; 75 μM of KCl; 20 μM of $MgSO_4$; 9 μM of substrate. Additional sucrose was also present in order to give a medium isotonic with that used in the measurement of oxidative phosphorylation. Final volume was 3.0 ml. In-

[1] A pirouette centrifuge (Phywe A. G., Göttingen, Germany) running in a cold room at 0°C. was used.

organic orthophosphate was determined in the trichloroacetic filtrates after stopping the reaction at zero time and after 30 minutes of incubation at 25°C. A control for the spontaneous hydrolysis of the substrate was also included. 0.5 ml. of 85 per cent (*w/v*) trichloroacetic acid was used for each tube and the contents were made up to 5.0 ml. with water before centrifuging and filtering.

Analytical Methods.—Inorganic orthophosphate was estimated by the method of Fiske and SubbaRow (16). Nitrogen was determined with Nessler's reagent after digestion of the samples with sulfuric acid containing copper selenite (17).

Morphological Examinations.—Sections stained with hematoxylin and eosin were carried out as routine to ascertain the cloudy swelling of the liver. Morphological examinations of isolated mitochondria were made with the koristka phase contrast microscope.

Reagents.—Cytochrome *c* was prepared from horse heart according to Keilin and Hartree (18). Other special chemicals were obtained commercially: a hexokinase preparation (lot H 124-56, found to have 3.2 units (HK)[2]/mg. under the conditions of Bailey and Webb (19)); adenosine-5'-phosphoric acid and dibarium adenosinetriphosphate (Sigma Chemical Co.); barium glucose-6-phosphate (Schwarz Laboratories, Inc.); α-ketoglutaric acid (General Biochemicals Inc.). All the other substances used were reagent grade.

Statistical Analysis.—Methods and terminology used in the statistical analysis are those given by Fisher (20).

RESULTS

Morphology

The examination of liver sections stained with hematoxylin and eosin confirmed that a typical cloudy swelling developed in the livers of all the animals which were treated with bacterial toxins.

Morphological differences between the mitochondria from the treated and the untreated animals could not be detected with certainty when isolated mitochondria were examined by phase contrast microscopy. This result is only apparently inconsistent with previous observations on tissue sections showing that changes in morphology of mitochondria occur during cloudy swelling (4, 6). The change of shape of mitochondria from rods to spheres, which has been observed in swollen cells, cannot be appreciated by examining mitochondria isolated in 0.25 M sucrose, a medium in which mitochondria from normal cells also appear spherical. On the other hand, only very marked changes in size can be appreciated by microscopic examination. Thus, the only assertion that is justified is that the mitochondria from treated animals used in the biochemical investigations were not much more swollen than the mitochondria from normal animals.

Biochemical Investigations

Using succinate and α-ketoglutarate as substrates, the oxidative phosphorylation in liver mitochondria from rats treated with *S. typhi murium* toxin and from guinea pigs treated with diphtheria toxin was compared with the behavior of mitochondria from the corresponding normal animals.

[2] HK is the symbol used by Bailey and Webb (19) to indicate their hexokinase unit.

The results in Table I show that the amount of inorganic orthophosphate which disappeared during the oxidation of either of the substrates was decreased in the mitochondria from treated animals as compared with normal controls, although for each substrate the oxygen consumption was practically the same whether the animals had been treated or not. Thus, the P:O ratios for both succinate and α-ketoglutarate were lower in mitochondria from treated animals than in normal mitochondria. The lowering of the phosphorylation quotients was statistically significant in either rats treated with *S. typhi murium* toxin ($P < 0.01$) or guinea pigs treated with diphtheria toxin ($P < 0.01$). In guinea

TABLE I

Oxidative Phosphorylation in Liver Mitochondria from Normal and Treated Animals

20 minutes of incubation at 25°C. with air as gas phase. Values referred to 1 mg. of mitochondrial N. The figures are means of all experiments; the sign ± precedes the standard error.

Animal	Substrate	Normal				Treated			
		No. of experiments	Inorganic orthophosphate disappeared	Oxygen consumed	P:O	No. of experiments	Inorganic orthophosphate disappeared	Oxygen consumed	P:O
			μM	μatoms			μM	μatoms	
Rat	Succinate	5	25.2	14.0	1.8 ± 0.07	5	19.9	14.8	1.3 ± 0.09
"	α-Ketoglutarate	10	10.7	3.4	3.2 ± 0.07	10	8.9	3.6	2.5 ± 0.16
"	α-Ketoglutarate*								
	Without malonate	4	11.4	3.6	3.2 ± 0.17	4	9.7	3.8	2.5 ± 0.17
	With malonate	4	11.1	2.8	4.0 ± 0.07	4	7.1	2.6	2.7 ± 0.29
Guinea pig	Succinate	5	18.7	9.5	2.0 ± 0.08	5	15.7	9.3	1.7 ± 0.07
	α-Ketoglutarate	4	7.0	2.3	3.1 ± 0.21	4	5.0	3.0	1.7 ± 0.14

* The same preparation of mitochondria was tested in the absence and presence of 0.02 M malonate (final concentration).

pigs but not in rats the effect of the treatment on the P:O ratio was greater using α-ketoglutarate as substrate than using succinate ($P < 0.01$).

In some α-ketoglutarate experiments with rat liver mitochondria, duplicates were carried out which were different only for the addition of 0.02 M malonate to the medium (*cf.* Table I). The purpose of these experiments was to study the generation of high energy phosphate bonds coupled with a single oxidative step and malonate has been largely used to obtain the one-step oxidation of α-ketoglutarate (21). In normal mitochondria, as to be expected, malonate depressed the oxygen consumption without affecting the inorganic orthophosphate disappearance. This resulted in an increase of the average P:O ratio from 3.2 to 4.0 (25 per cent). With mitochondria from treated rats, both the oxygen consumption and the inorganic orthophosphate disappearance were depressed. In this

case malonate enhanced very slightly the P:O ratio (from 2.5 to 2.7 = 8 per cent). The lowering of the P:O ratio for α-ketoglutarate, which was significant ($P < 0.01$) in mitochondria from treated rats as compared with normal controls, appeared therefore more evident when malonate was present in the medium.

Evidence has been presented (*cf.* Table I) that the amount of inorganic orthophosphate disappearing during the oxidation of succinate and α-ketoglutarate was lower in mitochondria from livers showing cloudy swelling than in normal liver mitochondria. This, however, did not necessarily mean that phosphorylation was inhibited in mitochondria from treated animals. The possibility existed that, in mitochondria from treated animals, the hydrolysis of phosphate esters during the incubation was increased, this also leading to a decreased inorganic orthophosphate disappearance. Fluoride added to the medium only partially inhibits the activity of phosphatases; that is, it reduces but does not eliminate the interference of dephosphorylating activities in the measurement of oxidative phosphorylation. On the other hand, a stimulation of the latent ATPase activity of mitochondria has been largely observed as a consequence of several treatments which damage the mitochondria and uncouple oxidative phosphorylation (22–29). Some increase of the ATPase activity has also been observed, together with uncoupling of oxidative phosphorylation, in mitochondria from fatty liver, a condition in which mitochondria appear morphologically changed (25). Experiments were then carried out to investigate the hydrolysis of some phosphate esters by liver mitochondria from normal and treated animals. A comment should be made at this point as to the conditions under which the dephosphorylating activities were studied. The activity of phosphatases is generally measured under conditions which are optimal for the activity of the enzyme; that is, under conditions very different from those used in phosphorylation experiments. Since the aim of our experiments was to ascertain the interference of the dephosphorylating activities in the phosphorylation measurements, we estimated the activity of phosphatases under conditions as close as possible to those used in phosphorylation experiments (*cf.* Materials and Methods). The hydrolysis of the following phosphate esters was studied: AMP, which was added as phosphate acceptor in the phosphorylation medium, ATP and G-6-P, which were formed during the phosphorylation reaction. Dephosphorylation of ATP was not significantly modified ($P > 0.1$) in liver mitochondria from treated animals, as compared with normal controls. The following figures were found for the hydrolysis of ATP (means of eight experiments ± standard error): *Rats*: normal, 20.8 ± 2.55; treated, 18.5 ± 1.94; *guinea pigs*: normal, 14.3 ± 1.90; treated, 9.6 ± 2.52 μM of inorganic orthophosphate liberated in 30 minutes at 25°C. per mg. of mitochondrial N. Under the conditions employed, no dephosphorylation of AMP and G-6-P was observed either in normal mitochondria or in the mitochondria from treated animals. Therefore, it appears justified to consider the decrease in inorganic orthophosphate disappearance in mitochondria from treated animals as the result of inhibited phosphorylation.

DISCUSSION

It has been shown in a previous paper (11) that a decrease of easily hydrolyzable phosphate occurs in tissues during cloudy swelling. Such a change appears well understandable in the light of the present findings in isolated mitochondria. The results presented show clearly that phosphorylation coupled with the oxidation of either succinate or α-ketoglutarate is inhibited in mitochondria isolated from livers showing cloudy swelling. This has been found in mitochondria from both rats injected with *S. typhi murium* toxin and guinea pigs treated with diphtheria toxin. However, there is some difference depending upon the toxin used to produce cloudy swelling. Phosphorylation associated with the oxidation of α-ketoglutarate is more affected by diphtheria toxin treatment than phosphorylation associated with the oxidation of succinate. On the contrary, the treatment with *S. typhi murium* toxin seems to affect to the same extent the two phosphorylating systems.

A close relationship seems to exist between cloudy swelling and the uncoupling of oxidative phosphorylation. It has already been mentioned that such a cellular change can be produced by injecting the animals with uncoupling agents such as 2,4-dinitrophenol (12) and thyroxine (13). On the other hand, uncoupling of oxidative phosphorylation has been found during this study in mitochondria isolated from livers showing cloudy swelling.

It is to be noted that cloudy swelling is the earliest histological evidence of cell degeneration, in which the alteration of mitochondria probably represents the most significant morphological change. Mitochondria are notoriously sensitive to unfavorable conditions of cell life and the specificity of any change in them is therefore open to question. It is significant in this regard that cloudy swelling can be observed in the cells as a response to a number of injuries; either it represents a transition stage towards more severe cell alterations or it is the only change that occurs.

It seems possible that the various injuries producing cloudy swelling have in common an inhibiting action on oxidative phosphorylation by damaging the mitochondria. The coupling between respiration and phosphorylation is a particularly labile mechanism and it is the first to suffer when mitochondria are in someway injured. Thus, it is tempting to suppose that cloudy swelling represents the morphological equivalent of the uncoupling between oxidation and phosphorylation.

SUMMARY

Using succinate and α-ketoglutarate as substrates, oxidative phosphorylation has been measured in mitochondria isolated from livers showing cloudy swelling. This cellular change was obtained by injecting rats with *S. typhi murium* toxin and guinea pigs with diphtheria toxin. It has been found that phosphorylation associated with the oxidation of either of these substrates was partially inhibited

in mitochondria from livers showing cloudy swelling, while the oxygen consumption was unchanged. Thus, the P:O ratios for both succinate and α-ketoglutarate were lower in mitochondria from treated animals than they were in normal mitochondria. Dephosphorylation of ATP was not significantly modified in mitochondria from livers showing cloudy swelling as compared with normal controls. No dephosphorylation of AMP and G-6-P was observed either in normal mitochondria or in mitochondria from treated animals.

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BIBLIOGRAPHY

1. Anitschkow, N., *Verhandl. deutsch. path. Ges.*, 1914, **17,** 103.
2. Dibbelt, W., *Verhandl. deutsch. path. Ges.*, 1914, **17,** 114.
3. Opie, E. L., *J. Exp. Med.*, 1947, **86,** 45.
4. Popják, G., *J. Path. and Bact.*, 1948, **60,** 75.
5. Zollinger, H. U., *Rev. hêmatol.*, 1950, **5,** 696.
6. Fonnesu, A., *Arch. sc. biol.*, 1954, **38,** 1.
7. Lehninger, A. L., *in* Phosphorus Metabolism. A Symposium on the Role of Phosphorus in the Metabolism of Plants and Animals, (W. D. McElroy and B. Glass, editors), Baltimore, The John Hopkins Press, 1951, **1,** 344.
8. Harman, J. W., and Feigelson, M., *Exp. Cell Research*, 1952, **3,** 47.
9. Harman, J. W. and Feigelson, M., *Exp. Cell Research*, 1952, **3,** 509.
10. Fonnesu, A., and Severi, C., *Boll. soc. ital. biol. sper.*, 1952, **28,** 1923.
11. Fonnesu, A., and Severi, C., *Gior. biochim.*, 1953, **2,** 326.
12. Fonnesu, A., and Severi, C., *Arch. sc. biol.*, 1954, **38,** 597.
13. Fonnesu, A., and Severi, C., data to be published.
14. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114,** 495.
15. Potter, V. R., *J. Biol. Chem.*, 1947, **169,** 17.
16. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66,** 375.
17. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Tissue Metabolism, Minneapolis, Burgess Publishing Co., 1949.
18. Keilin, D., and Hartree, E. F., *Biochem. J.*, 1945, **39,** 289.
19. Bailey, K., and Webb, E. C., *Biochem. J.*, 1948, **42,** 60.
20. Fisher, R. A., Statistical Methods for Research Workers, Edinburgh, Oliver and Boyd, 11th edition, 1950.
21. Copenhaver, J. H., and Lardy, H. A., *J. Biol. Chem.*, 1952, **195,** 225.
22. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, 1951, **191,** 485.
23. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, 1953, **201,** 357.
24. Potter, V. R., Siekevitz, P., and Simonson, H. C., *J. Biol. Chem.*, 1953, **205,** 893.
25. Dianzani, M. U., *Biochim. et biophysic. Acta*, 1954, **14,** 514.
26. Hunter, F. E., Jr., *in* Phosphorus Metabolism. A Symposium on the Role of Phosphorus in the Metabolism of Plants and Animals, (W. D. McElroy and B. Glass, editors), Baltimore, The John Hopkins Press, 1951, **1,** 297.
27. Harman, J. W., and Kitiyakara, A., *Exp. Cell Research*, 1955, **8,** 411.
28. Witter, R. F., Newcomb, E. H., and Stotz, E., *J. Biol. Chem.*, 1953, **202,** 291.
29. Kaltenbach, J. C., and Harman, J. W., *Exp. Cell Research*, 1955, **8,** 435.