Effect of Phlorizin on the Osmotic Behavior of Mitochondria in Isotonic Sucrose*

DANIEL M. KELLER† AND WILLIAM D. LOTSPEICH

From the Department of Physiology, College of Medicine, University of Cincinnati, Cincinnati, Ohio

(Received for publication, October 27, 1958)

Although phlorizin appears clearly capable of inhibiting respiration and diminishing the efficiency of oxidative phosphorylation (1), the possibility remains that these effects are secondary to a general nonspecific alteration of mitochondrial structure rather than to a single specific inhibition somewhere in the chain of enzymes and coenzymes concerned in oxidative metabolism. For instance, the inhibition of oxidative phosphorylation by the hormone thyroxine, or its metabolites, has been related to some such general alteration of mitochondrial structure (2). Such a structural effect of phlorizin would not only explain its inhibition of mitochondrial metabolism but might harmonize those observations with the known effect that phlorizin has on certain cell membranes.

The studies reported here deal with the osmotic behavior of mitochondria in isotonic sucrose and show that phlorizin promotes the swelling of mitochondria in such an isotonic medium. Furthermore, it shows that ATP and ADP, but not AMP, prevent this phlorizin-induced swelling. These observations are then related to the effects of adenine nucleotides, malonate, 2,4-dinitrophenol, and electron carrier steady state on mitochondrial structure and the phlorizin inhibition of respiration.

EXPERIMENTAL

Since the first observation by Claude (3) that mitochondria swell when placed in hypotonic media, several techniques have been used to study the phenomenon. The changes in mitochondrial structure may be revealed by directly observing the swelling under the phase contrast microscope (4), by a reduction of the dry weight to wet weight ratio (5), or by a decrease in the optical density of the mitochondrial suspensions (6, 7).

In our experiments changes in mitochondrial volume were studied at room temperature by following the optical density changes at 520 mμ in the Beckman model DU spectrophotometer with the use of a technique essentially like that described by Tapley (8). The cuvettes contained 3 ml. consisting of mitochondria, 0.3 M sucrose, and 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.4. Except where indicated, experiments were performed with mitochondria prepared from guinea pig kidney cortex according to the method of Dounce *et al.* (9) which involves 0.001 M ethylenediaminetetraacetate during homogenization and the first washing and a medium of 0.44 M sucrose throughout. Before addition to the spectrophotometer cuvette the final mitochondrial pellet was suspended in sufficient volume of 0.44 M sucrose so that 0.1 ml. of suspension in a final volume of 3 ml. would give an optical density of about 0.5. The mitochondria were used immediately after their preparation.

* Supported by a grant from the Life Insurance Medical Research Fund of New York.

† This work was performed during the tenure of a Life Insurance Medical Research Fund Postdoctoral Fellowship.

RESULTS

Effect of Sucrose Tonicity on Mitochondrial Volume—As may be seen in Fig. 1, guinea pig kidney mitochondria swell very rapidly when placed in hypotonic sucrose so that by the time the first reading is taken at 1 minute swelling is already complete. A rapid swelling in hypotonic media was also found by Cleland (10) for rat heart sarcosomes. However, this may be contrasted to the behavior of rat liver mitochondria which swell slowly over a 10- to 20-minute interval under these conditions (8).

Effect of Phlorizin on Mitochondrial Volume in Isotonic Sucrose—The results from respiration experiments on whole homogenates and respiring mitochondria (1) suggested that phlorizin may cause a general alteration in mitochondrial structure. The curves in Fig. 2 show that although there is no swelling of mitochondria in control vessels, phlorizin causes a definite swelling in the 0.3 M sucrose. Determination of the dry weight to wet weight ratio showed that the decrease in optical density initiated by phlorizin was associated with increase in water content of the mitochondria and thus represented a true swelling from water imbibition. This type of swelling can be distinguished from that induced in hypotonic solutions, because it has a much longer time course. The dose-response relationship for the phlorizin-induced swelling phenomenon is quite comparable to that which exists for the phlorizin-induced block of oxidation; that is, perceptible at 2×10^{-4} M and marked at 10^{-3} M. Furthermore, it was found that phlorizin could initiate additional swelling even in mitochondria which were already severely swollen in a hypotonic medium of 0.06 M sucrose.

Effect of Adenine Nucleotides on Phlorizin-induced Swelling—In view of the finding (1, 11) that sufficient concentrations of ATP can reduce the phlorizin effect on oxidation, it was of particular interest to test the effect of ATP on phlorizin-induced swelling in the mitochondria. In Fig. 3 are shown results of experiments of this type in which it was found that 5×10^{-3} M ATP in the isotonic sucrose medium effectively blocks the swelling induced by 10^{-3} M phlorizin. It has been shown that ATP or the onset of oxidative phosphorylation or both can, under certain conditions, bring about a contraction of swollen mitochondria (12). This might be the basis for the adenine nucleotide reversal of the phlorizin inhibition of respiration in kidney tissue (1). However, as is evident in the experiment of Fig. 4, under the conditions of the present swelling studies, ATP could

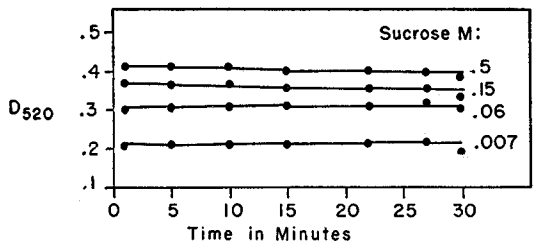

FIG. 1. Effect of sucrose concentration on swelling of guinea pig kidney mitochondria. The only other component of the medium was 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.4. Optical density read against air.

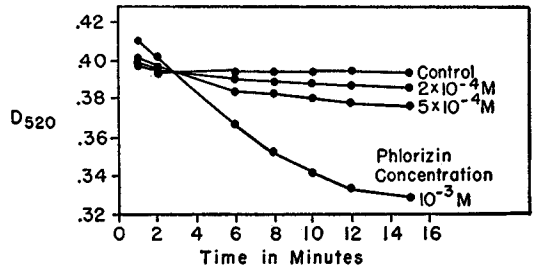

FIG. 2. Swelling of guinea pig kidney mitochondria induced by 2×10^{-4}, 5×10^{-4}, and 10^{-3} M phlorizin in 0.3 M sucrose buffer medium. Optical density read against air.

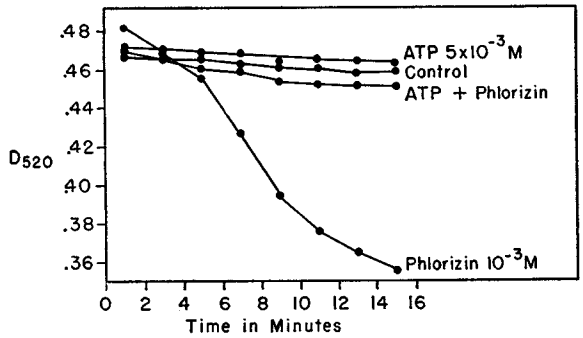

FIG. 3. Effect of ATP on phlorizin-induced mitochondrial swelling in 0.3 M sucrose buffer medium. Optical density read against air.

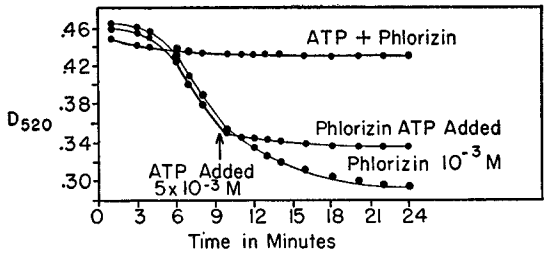

FIG. 4. Effect of added ATP on the course of phlorizin-induced mitochondrial swelling. Sucrose buffer medium, 0.3 M. Optical density was read against a blank and correction made for the dilution caused by adding ATP solution at 9.5 minutes.

only stop the progression of phlorizin-induced swelling and could not completely reverse it and return the mitochondria to their former volume. This observation is in agreement with Tapley's finding (8) that ATP could stop, but not completely reverse, the hypotonicity-induced swelling of rat liver mitochondria.

Comparison of Effects of ATP, ADP, and AMP on Phlorizin

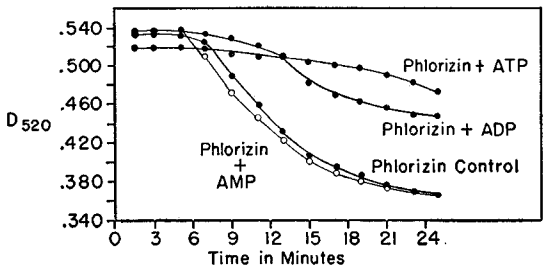

FIG. 5. A comparison of ATP, ADP, and AMP at low concentration, 2×10^{-4} M, on phlorizin-induced mitochondrial swelling. Guinea pig kidney mitochondria suspended in 0.3 M sucrose buffer medium. Optical density read against air.

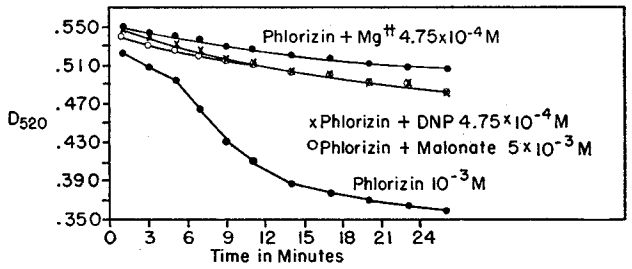

FIG. 6. Effect of malonate, 2,4-dinitrophenol, and magnesium ion on phlorizin-induced mitochondrial swelling. Guinea pig kidney mitochondria suspended in 0.3 M sucrose buffer medium. Optical density read against air.

Swelling in Mitochondria—When it was seen that ADP (5×10^{-3} M) was as effective as ATP in blocking phlorizin-induced swelling (Fig. 4), an attempt was made to reconcile this observation with the finding (1) that mitochondria in a respiratory medium with ADP (State II) are much more susceptible to phlorizin than when they are in a respiratory medium with ATP (State IV). It seemed possible that ADP was acting under the conditions used here by being converted to ATP and AMP by adenylate kinase (13) or in some other way making its high energy phosphate available. In Fig. 5 are plotted data which lend support to this possibility. It may be seen from this experiment that low concentrations of ATP (2×10^{-4} M) forestalled swelling for approximately 15 minutes after the control mitochondria began to swell, whereas ADP in equal concentration (but, of course, containing one half as much high energy phosphate) prevented swelling for only about half as long. And finally, in contrast to the other two adenine nucleotides, AMP, which contains no high energy phosphate, was incapable of preventing the phlorizin-induced swelling.

Effect of Malonate, Dinitrophenol, and Mg^{++}—It has been indicated in the previous paper (1) that 20×10^{-3} M malonate can reduce the effect of phlorizin on oxidative metabolism whereas the phlorizin effect was unaffected by 10^{-4} M dinitrophenol. Both of these agents are known to reduce the swelling of rat liver mitochondria under various conditions (6, 8) so that it became of interest to study their effect, as well as that of phlorizin, on the mitochondrial swelling phenomenon. Fig. 6 is from an experiment which demonstrates that 5×10^{-3} M malonate and 4.75×10^{-4} dinitrophenol, like ATP, are equally effective in counteracting the phlorizin-induced swelling phenomenon. In addition, it may be seen that magnesium ion, an agent which inhibits mitochondrial swelling under other conditions (6), also blocks the phlorizin-induced swelling in isotonic sucrose.

Effect of Phlorizin on Mitochondrial Volume in Respiration Medium—The data presented thus far show good correlation between the dose-response relation for swelling and for inhibition of oxidation, and in addition there is a similarity between the effects of ATP and malonate on both processes. Furthermore, it had been observed in other experiments that phlorizin can promote swelling and that ATP can prevent it even when the 0.3 M sucrose of the medium was replaced by 0.3 M glucose, 0.15 M KCl, or 0.1 M potassium α-ketoglutarate. For these reasons it was decided to study phlorizin-induced swelling in the same medium that had been used in the respiration experiments of the previous study (1) in which a variety of interactions might alter the results considerably.

Fig. 7 is a plot of optical density measurements against time for two such experiments. The experimental conditions for *A* and *B* correspond approximately to the conditions for State II and State IV mitochondrial respiration experiments respectively (1). However, fewer mitochondria were present here to allow for optical density measurements, and the incubation was carried out in the cuvettes of the spectrophotometer at room temperature rather than in the microrespiration flasks. Rabbit kidney mitochondria prepared by the method of Schneider (14) were used. The readings were taken against mitochondrial blanks to allow correction to be made for decrease in optical density due simply to the mitochondrial dilution that occurred with each addition.

It may be seen that upon adding phlorizin in the experiment of *A* (State II) a prompt decrease in optical density occurred. In contrast to this, the addition of phlorizin in the experiment of *B* (State IV) did not change the optical density from the control cuvette. This finding correlates well with the observation (1) that phlorizin inhibits respiration strongly when added to State II mitochondria, but that little or no inhibition occurs when added to State IV. However, the good correlation ends upon conversion to State III by adding substrate in *A* and hexokinase-glucose in *B*. Perhaps the failure of the phlorizinized mitochondria in *B* to behave as the nonphlorizinized, as was the case in respiration experiments, was the result of the unavoidable differences between the experimental conditions used here and those used in the respiration studies.

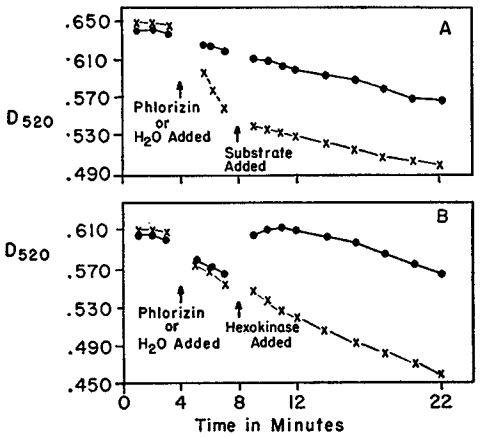

FIG. 7. Effect of phlorizin on optical density of rabbit kidney mitochondria suspended in respiration medium. *A*, phlorizin 10^{-3} M added to mitochondria in presence of ADP and hexokinase (State II). *B*, phlorizin added to mitochondria in presence of ATP and substrate (α-ketoglutarate) (State IV). Readings were taken against a blank and corrections made for dilution of the suspensions. ●, control; ×, phlorizin.

DISCUSSION

Phlorizin in concentrations of 5×10^{-4} to 10^{-3} M can inhibit oxidation and reduce the efficiency of phosphorylation in mitochondria (1). It is believed on the basis of the studies presented above that this inhibition is probably the result of a general structural alteration of mitochondria rather than a specific single enzyme effect. It has been suggested (15) that the maintenance of mitochondrial structure depends upon some active principle in its enzymatic organization. If this is so, phlorizin may act to disrupt and adenine nucleotide to preserve this function. That the metabolism-inhibiting and structure-altering effects are the result of the same action of phlorizin is indicated by the similar dose response range for both effects and the fact that both are diminished by ATP, malonate, and the steady state metabolic conditions for State IV.

Both swelling and decreased efficiency of oxidative phosphorylation occur in the so called aging process (15) of mitochondria, and it seems probable that phlorizin should be grouped along with the several other agents that have been demonstrated to initiate this process. The conclusion that phlorizin acts in this way could be substantiated by testing for the appearance of other phenomena which occur in aging, such as the decreased ability to concentrate certain ions (5) and nucleotides (16), diminished phosphate-oxygen exchange (17), appearance of DPNase (18) and of a magnesium-dependent ATPase (19).

The possible relevance of these findings to phlorizin action *in vivo* is indicated by the report of Nagai (20) who showed that kidney mitochondria from animals chronically treated with phlorizin have a reduced efficiency of oxidative phosphorylation. Von Kossa, quoted by Lusk (21), found severe cloudy swelling of the convoluted tubules of kidneys from rabbits treated with phlorizin. In view of the present interpretation that cloudy swelling is actually due to mitochondrial swelling, this probably indicates that phlorizin, in sufficient concentration, can cause mitochondrial swelling *in vivo*.

Does phlorizin reach concentrations in the proximal tubule cell sufficient to inhibit oxidative metabolism? In a recent study (22) it has been shown that glucose reabsorption is exquisitely sensitive to phlorizin. A total cumulative dose of 2.8 mg. of phlorizin in a 20-kg. dog over a 70-minute period resulted in more than 50 per cent reduction in the tubular capacity to transport glucose. The approximate concentration of phlorizin that has been shown to inhibit oxidative metabolism by 50 per cent *in vitro* is 5×10^{-4} M (1, 11, 23). In order for 2.8 mg. to have this concentration in the renal tubular cells transporting glucose, it would have to occupy a volume of 11.9 ml. Although this volume is probably somewhat larger than the total volume of the cells of the proxmial convoluted tubule, it is obvious that these cells would have to have a very high affinity for phlorizin so that its concentration in other tissues would be many fold lower. Ellinger and Lambrechts (24) have shown that colored glucosuric derivatives of phlorizin are, indeed, concentrated in the cells of the proximal tubule. But it must be remembered that some of the phlorizin administered to the dog was no doubt lost through excretion by the kidneys (25) and liver (26). This argument indicates that a phlorizin concentration of 5×10^{-4} M in the cells of the proximal tubule is in the realm of possibility but that a direct measure of phlorizin concentration here is certainly needed.

SUMMARY

It has been shown that phlorizin in concentrations comparable to those which inhibit oxidation in mitochondria also cause swelling of mitochondria in isotonic sucrose. Phlorizin is less able to induce swelling in the presence of adenosine triphosphate, adenosine diphosphate, and malonate, and under the metabolic conditions that obtain when phlorizin is added to mitochondria in the presence of ATP and α-ketoglutarate. These effects on phlorizin-induced swelling correlate well with the effects of these agents and conditions on the phlorizin inhibition of oxidation. It thus appears that the phlorizin effect on mitochondrial metabolism may be secondary to a general effect on mitochondrial structure exerted most probably at the membrane.

REFERENCES

1. KELLER, D. M., AND LOTSPEICH, W. D., *J. Biol. Chem.*, **234,** 987 (1959).
2. TAPLEY, D. F., AND COOPER, C., *J. Biol. Chem.*, **222,** 341 (1956).
3. CLAUDE, A., *J. Exptl. Med.*, **84,** 51 (1946).
4. HARMAN, J. W., AND FEIGELSON, M., *Exptl. Cell Research*, **3,** 47 (1952).
5. PRICE, C. A., FONNESU, A., AND DAVIES, R. E., *Biochem. J.*, **64,** 754 (1956).
6. RAAFLAUB, J., *Helv. Physiol. Acta*, **11,** 142 (1953).
7. MALAMED, S., AND RECKNAGEL, R. O., *Proc. Soc. Exptl. Biol. Med.*, **98,** 139 (1958).
8. TAPLEY, D. F., *J. Biol. Chem.*, **222,** 325 (1956).
9. DOUNCE, A. L., WITTER, R. F., MONTY, K. J., PATE, S., AND COTTONE, M. A., *J. Biophys. Biochem. Cytol.*, **1,** 140 (1955).
10. CLELAND, K. W., *Nature*, **170,** 497 (1952).
11. LOTSPEICH, W. D., AND KELLER, D. M., *J. Biol. Chem.*, **222,** 843 (1956).
12. CHAPPELL, J. B., AND PERRY, S. V., *Nature*, **173,** 1094 (1954).
13. KIELLEY, W. W., AND KIELLEY, R. K., *J. Biol. Chem.*, **191,** 485 (1951).
14. SCHNEIDER, W. C., *J. Biol. Chem.*, **176,** 259 (1948).
15. ERNSTER, L., AND LINDBERG, O., *Annual Review of Physiology, Vol. 20*, Annual Reviews, Inc., Palo Alto, California, 1958, p. 13.
16. SIEKEVITZ, P., AND POTTER, V. R., *J. Biol. Chem.*, **215,** 221 (1955).
17. BOYER, P. D., LUCHSINGER, W. W., AND FALCONE, A. B., *J. Biol. Chem.*, **223,** 405 (1956).
18. HUNTER, F. E., JR., AND FORD, L., *J. Biol. Chem.*, **216,** 357 (1955).
19. POTTER, V. R., SIEKEVITZ, P., AND SIMONSON, H. C., *J. Biol. Chem.*, **205,** 893 (1953).
20. NAGAI, K., *Med. J. Osaka Univ.*, **6,** 907 (1956).
21. LUSK, G., *Ergeb. Physiol.*, **12,** 315 (1912).
22. LOTSPEICH, W. D., AND WORONKOW, S., *Am. J. Physiol.*, **195,** 331 (1958).
23. SHAPIRO, B., *Biochem. J.*, **41,** 151 (1947).
24. ELLINGER, P., AND LAMBRECHTS, A., *Compt. rend. soc. biol.*, **124,** 261 (1937).
25. BRAUN, W., WHITTAKER, V. P., AND LOTSPEICH, W. D., *Am. J. Physiol.*, **190,** 563 (1957).
26. JENNER, F. A., AND SMYTH, D. H., *J. Physiol.*, **137,** 18P (1957).