Some Effects of Phlorizin on the Metabolism of Mitochondria*

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The exact nature of the phlorizin inhibition of biological transport remains unknown. A number of different studies have shown effects of the glycoside on both membrane permeability (1, 2) and cellular metabolism (3, 4). Shapiro (3) found in 1947 that phlorizin in concentrations of 2×10^{-4} to 10^{-3} M reduces the aerobic utilization of glucose, pyruvate, and citrate and depresses creatine phosphorylation in a rat kidney mince. These observations were extended by Lotspeich and Keller (4) to homogenates of guinea pig kidney. They demonstrated in this preparation that phlorizin in similar concentrations inhibits the oxidation of all the substrates of the tricarboxylic acid cycle and that this inhibition can be completely prevented by an excess of adenine nucleotides.

Since the mitochondria contain the enzymes of the tricarboxylic acid cycle and associated oxidative phosphorylation, it seemed worthwhile to extend our studies to these subcellular structures. Accordingly the present paper presents experiments with isolated respiring mitochondria from both kidney and liver. In these organelles phlorizin produces a similar defect of oxidative metabolism which has a special relation to the steady state of the respiratory electron carrier system. Besides reporting these studies, this paper also serves to introduce the following one (5) on the effect of phlorizin on the swelling of mitochondria in isotonic sucrose. These experiments, we believe, largely explain the phlorizin defect in oxidative metabolism and also harmonize the disparate observations implicating phlorizin in both oxidative metabolism and membrane permeability.

EXPERIMENTAL

Studies on oxidative phosphorylation were carried out essentially as described by Copenhaver and Lardy (6). To the main compartment of the Warburg flasks the various components were added from isotonic solutions to give the following final concentrations in a 3-ml. volume: potassium phosphate buffer, pH 7.3, 13.3 mM; substrate (succinate, L-malate, β-hydroxybutyrate, or α-ketoglutarate), 6.7 mM; ATP, 2 mM; $MgSO_4$, 7.5 mM; cytochrome *c*, 0.012 mM; 0.5 ml. of mitochondria from 0.5 gm. of tissue; 0.25 M sucrose to a volume of 2.7 ml. When disodium malonate was used to limit the oxidation of α-ketoglutarate to one step (6), its concentration was 20 mM. Phlorizin, when used, was placed in the main compartment unless otherwise noted. The hexokinase preparation, 0.2 ml., and glucose, 0.1 ml. (for a final concentration of 16.7 mM) were added to the side arm. Saturated KOH, 0.2 ml., and a filter paper strip were placed in the center well. Triplicate flasks were incubated at 30° with air as the gas phase. After 3 minutes the side arm contents were tipped into the main compartment, and at 5 minutes the initial manometer readings were taken for oxygen uptake over the next 8 to 20 minutes. Deproteinization with 5 per cent trichloroacetic acid was carried out on one flask of each of triplicates at 5 minutes and on the respiration flasks immediately after the final manometer reading. Inorganic phosphate was determined by the method of Lowry and Lopez (7), and phosphate uptake was calculated as the difference between that remaining after respiration and that present in the flask deproteinized at 5 minutes. The P:O ratio was calculated as the ratio of the micromoles of inorganic phosphate taken up to the microatoms of oxygen used.

Mitochondria were prepared from rabbit kidney cortex (or other tissue when specified) in 0.25 M sucrose according to the method of Schneider (8). Each milliliter of final suspension represented 1 gm. of original tissue. Crude hexokinase was prepared by a method of Loomis (unpublished)[1] and contained about 300 Kunitz-McDonald units per ml. (9).

Cytochrome *c* and the sodium salts of ATP and ADP were obtained from the Sigma Chemical Company. Crystalline phlorizin was obtained from Bios Laboratories, Inc. This material was strongly glycosuric, showed a single spot with the R_F of phlorizin on the paper chromatogram (10), and showed the characteristic ultraviolet absorption peak of phlorizin at 285 mμ in the Beckman spectrophotometer.

RESULTS AND DISCUSSION

Studies on Efficiency of Oxidative Phosphorylation—An initial series of experiments, summarized in Table I, was done to test for a phlorizin effect on oxygen uptake and P:O ratio. Mitochondria from three tissues (rat liver, guinea pig kidney cortex, rabbit kidney cortex) were tested, and four different substrates were used (succinate, L-malate, β-hydroxybutyrate, and α-ketoglutarate with malonate). In every instance where phlorizin was present at 5×10^{-4} M or higher, oxygen utilization was inhibited and the efficiency of phosphorylation diminished. These results agree well with the report of Nagai (11) that mitochondria prepared from kidneys of rats given prolonged treatment with phlorizin showed lowered P:O ratios.

With these findings established, it was important to test the effect of phlorizin on the isolated hexokinase reaction, because an inhibition of phosphate transfer from ATP to glucose might conceivably account for the observed oxidative effects. This was done by using the Colowick and Kalckar (12) technique for

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[1] The method was kindly supplied to the authors by Dr. Roger L. Greif, Department of Physiology, Cornell University Medical College.

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TABLE I

Effect of phlorizin on oxidation and phosphorylation in mitochondria from kidney and liver

Experiment No.	Substrate	Phlorizin concentration	Oxygen Utilization		P:O ratio	
			Control	Phlorizin	Control	Phlorizin
		M	*μatoms/min./flask*			
1*	α-Ketoglutarate + malonate	5×10^{-4}	.526	.320	3.0	0.5
2†	α-Ketoglutarate + malonate	5×10^{-4}	.380	.140	2.4	2.2
3	α-Ketoglutarate + malonate	5×10^{-4}	.473	.260	2.7	1.8
4	α-Ketoglutarate + mlaonate	5×10^{-4}	.500	.327	2.8	2.4
5	α-Ketoglutarate + malonate	5×10^{-4}	.367	.220	2.4	1.3
6	α-Ketoglutarate + malonate	5×10^{-4}	.367	.307	2.4	2.4
7	α-Ketoglutarate + malonate	1×10^{-3}	.487	.307	2.2	1.3
8	α-Ketoglutarate + malonate	1×10^{-2}	.420	.033	3.0	0.5
9‡	α-Ketoglutarate + malonate	5×10^{-4}	.400	.390	3.1	3.0
10	α-Ketoglutarate + malonate	1×10^{-3}	.300	.187	2.1	0.9
11	α-Ketoglutarate + malonate	1×10^{-3}	.280	.167	3.0	2.7
12	α-Ketoglutarate + malonate	1×10^{-3}	.310	.210	3.9	2.9
13	α-Ketoglutarate + malonate	1×10^{-3}	.268	.193	3.2	2.9
14	β-Hydroxybutyrate	1×10^{-3}	.233	.180	1.5	1.3
15	β-Hydroxybutyrate	1×10^{-3}	.200	.153	2.1	1.9
16	β-Hydroxybutyrate	1×10^{-3}	.173	.153	2.4	2.0
17	β-Hydroxybutyrate	1×10^{-3}	.200	.180	2.4	1.9
18	Succinate	1×10^{-3}	.620	.340	1.9	1.4
19	Succinate	1×10^{-3}	.933	.587	1.9	1.5
20	L-malate	1×10^{-3}	.307	.187	2.6	1.7
21	L-malate	1×10^{-3}	.453	.300	2.2	2.1

* Mitochondria from rat liver.

† Experiments 2 through 8, mitochondria from guinea pig kidney cortex.

‡ Experiments 9 through 21, mitochondria from rabbit kidney cortex.

hexokinase assay. These experiments showed conclusively that phlorizin, in concentrations up to 3×10^{-3} M, had no effect on the reaction velocity of the hexokinase system.

Effect of Electron Carrier Steady State of Mitochondria on Phlorizin Inhibition—In the initial series of experiments (Table I) it was observed that the phlorizin inhibition was quite variable from one experiment to the next, and it soon became apparent that the ability of phlorizin to inhibit respiration was markedly affected by the order in which the reactants were added to the mitochondria.

The effects of ADP, substrate, and oxygen on the oxidation-reduction state of the electron carrier system in mitochondria have been studied by Chance and Williams (13). Freshly prepared mitochondria incubated aerobically without added substrate or ADP were defined by them as being in State I. If ADP was then added, a brief burst of respiration ensued but ended when all endogenous substrate had been utilized. This nonrespiring condition was called State II and was characterized by complete oxidation of the coenzymes in the electron carrier system. State III was obtained by adding substrate to State II mitochondria and was characterized by active respiration and partial reduction of all electron carrier coenzymes. If respiration continued until all ADP was converted to ATP, respiration slowed and State IV was obtained. This was characterized by complete reduction of DPN and complete oxidation of cytochrome *a* with partial reduction of the intermediate coenzymes.

Preliminary experiments had indicated that phlorizin might inhibit more effectively when added to State II than to State IV mitochondria, and it was decided to test this possibility. Warburg flasks with two side arms were used. State II mitochondria were prepared by placing them together with phosphate buffer, $MgSO_4$, cytochrome *c*, ADP, hexokinase, and glucose in the main compartment. Phlorizin was tipped in from the first side arm at 4 minutes and α-ketoglutarate, from the second side arm at 8 minutes. State IV mitochondria were prepared by placing them together with buffer, $MgSO_4$, cytochrome *c*, ADP, and α-ketoglutarate in the main compartment. Phlorizin was again in the first side arm, but the second side arm contained the hexokinase-glucose phosphate acceptor system. Of course, when the second side arm contents were tipped in, the mitochondria were in both cases converted to State III and active respiration occurred.

It may be seen from Table II that 10^{-3} M phlorizin added to mitochondria in State II caused severe inhibition of the respiration initiated by substrate addition, whereas phlorizon added to State IV mitochondria had much less or no effect on respiration occurring upon addition of hexokinase-glucose. It thus appears that mitochondria are much less susceptible to the effects of phlorizin when they are in State IV, *i.e.* when the electron carriers are in the partially reduced state and the adenine nucleotide is in the form of ATP.

Adenine Nucleotide Reversal of Phlorizin Inhibition—Whether the partially reduced state of the electron carriers, the ATP, or both protected the mitochondria could not be directly determined. However, it was decided to study further the effects of ATP for two reasons. (*a*) It had been reported by Lotspeich and Keller (4) that high concentrations of adenine nucleotides could prevent phlorizin inhibition of respiration in guinea pig kidney homogenates. (*b*) Tapley (14) and others have shown that ATP can prevent mitochondrial swelling in hypotonic me-

TABLE II

Effect of electron carrier steady state of mito on phlorizin inhibition

Experiment No.	Oxygen utilization			
	State II*		State IV†	
	Control	Phlorizin 10^{-3} M	Control	Phlorizin 10^{-3} M
	μatoms/min./flask		*μatoms/min./flask*	
1	.76	.37	.77	.75
2	.76	.37	.75	.72
3	.77	.36	.86	.71

* Hexokinase in center; phlorizin added at 4 minutes; α-ketoglutarate at 8 minutes.

† α-Ketoglutarate in center; phlorizin added at 4 minutes; hexokinase at 8 minutes.

dia. This second point will be dealt with in the following paper. The first point, relative to the ability of an excess concentration of adenine nucleotide to reduce the phlorizin inhibition, was verified in mitochondria, as shown in Table III. Control flasks and flasks with 5×10^{-4} M phlorizin were incubated with ATP at 2 mM and 10 mM concentrations. As can be seen, the phlorizin inhibition was reduced from 45 per cent at the low concentration to 23 per cent at the high concentration of ATP.

Thus in mitochondria, as in whole kidney homogenates (4), one can demonstrate the protection against phlorizin inhibition of respiration by excess adenine nucleotide. The experiments presented in the following paper show that ATP prevents the phlorizin-induced swelling of mitochondria in isotonic sucrose and raises the possibility that both the phlorizin inhibition of respiration and the adenine nucleotide reversal can be explained by a membrane permeability effect of phlorizin on mitochondria with resulting changes in the capacity of these structures to maintain normal osmoregulation. Thus the adenine nucleotide reversal of the phlorizin inhibition of respiration would be the result of a prevention of the phlorizin-induced swelling of mitochondria in an "isotonic" environment.

Effect of Malonate on Phlorizin Inhibition—Besides acting as a competitive inhibitor of succinic dehydrogenase, malonate has been shown, like ATP, to prevent the swelling of mitochondria in hypotonic media under certain conditions (14, 15). Since it seems possible, as is shown in the following paper, that the ATP reversal of the phlorizin effect on respiration correlates with the ATP reversal of phlorizin-induced swelling of mitochondria in isotonic sucrose, it was decided to study the effect of phlorizin on α-ketoglutarate oxidation in mitochondria in the absence and presence of malonate. In Table IV are shown the results of such experiments. In these experiments phlorizin was tipped in first from one side arm at 4 minutes; α-ketoglutarate and malonate, from the other side arm at 8 minutes; oxygen uptake measurements were begun at 12 minutes. It is evident from these experiments that the phlorizin inhibition of α-ketoglutarate oxidation in mitochondria is much less when malonate is present than in its absence. This observation may be related to the fact that malonate limits the oxidation of α-ketoglutarate to one step, *i.e.* to succinate. Alternatively, the fact that malonate protects mitochondrial structure and hence the enzymes of oxidative metabolism may bear on this difference.

Relation between Phlorizin Concentration and Oxidative Inhibition in Mitochondria—Fig. 1 is a plot of the per cent inhibition of oxygen consumption against phlorizin concentration when phlorizin is added to State II mitochondria. It is evident that the effective range of phlorizin concentrations here is very similar to that seen in the kidney mince (3) and the whole homogenate (4). In the mitochondria, after respiration commenced with the addition of substrate, there was a definite tendency for the respiratory rate to recover from the effects of phlorizin. This effect and its relation to phlorizin concentration are seen in Fig. 2 which is a plot of the per cent inhibition of oxygen utilization against time in minutes after onset of incubation at several different phlorizin concentrations. It appears that at the lowest concentration there is complete recovery of respiration within a few minutes as is seen in the bottom curve; at higher phlorizin concentrations there is only partial recovery or none at all. The capacity to recover from the effects of the inhibitor may be due to the production during respiration of a substance, perhaps ATP, which counteracts the effect of phlorizin. Or perhaps the respiratory enzymes are for

TABLE III

Reduction of phlorizin inhibition by ATP

Experimental conditions	Oxygen utilization		Inhibition by phlorizin
	Control	Phlorizin 5×10^{-4} M	
	μatoms/min./flask		%
ATP, 2 mM	.58	.32	45
ATP, 10 mM	.53	.41	23

TABLE IV

Effect of malonate on phlorizin inhibition of α-ketoglutarate oxidation by rabbit kidney mitochondria

For experimental details see text.

Experimental conditions	No. of experiments	Oxygen utilization		Per cent of control (mean ± standard error)
		Control	Phlorizin 10^{-3} M	
		μatoms/min./flask		
α-Ketoglutarate	6	.763	.340	44.7 ± 1.7
α-Ketoglutarate + malonate	11	.420	.302	71.6 ± 2.1

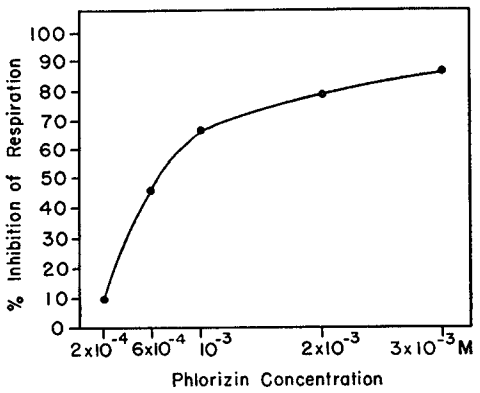

FIG. 1. Relation between phlorizin concentration and inhibition of respiration in kidney cortex mitochondria. α-Ketoglutarate was substrate.

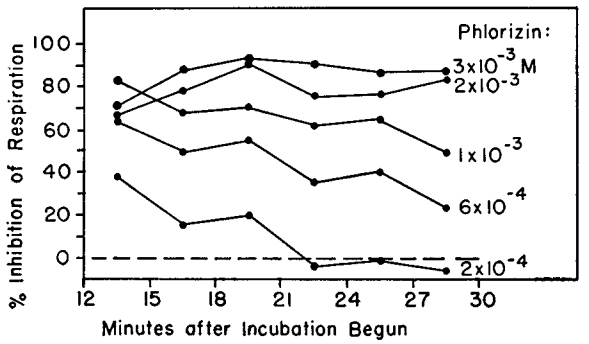

FIG. 2. Experiments showing the varying duration of respiratory inhibition in mitochondria with different phlorizin concentrations. α-Ketoglutarate was substrate.

TABLE V

Effect of phlorizin on oxygen utilization and P:O ratio with and without 2,4-dinitrophenol

Phlorizin added at 4 minutes; α-ketoglutarate and malonate at 8 minutes; oxygen uptake measured between 12 and 22 minutes.

Experimental conditions	Oxygen utilization		P:O	
	Control	Phlorizin 10^{-3} M	Control	Phlorizin 10^{-3} M
	μatoms/min./flask			
No DNP	.484	.376	3.54	2.50
DNP, 10^{-4} M	.420	.301	0.48	0.30

some reason less susceptible to the phlorizin effect when they are in the partially reduced state.

Relation between Phlorizin and 2,4-Dinitrophenol Effects on Oxidative Phosphorylation—Although it appeared that the phlorizin inhibition occurred somewhere in the series of reactions concerned with respiratory electron transfer or the coupled phosphorylations, its effect differed from that of the classical "uncoupler," 2,4-dinitrophenol, in several important respects. Although producing maximal uncoupling, dinitrophenol can stimulate rather than depress respiration; its reduction of the P:O ratio is much more marked than that caused by phlorizin (Table I), and finally the effects of dinitrophenol are not reversed by addition of any of the adenine nucleotides. For these reasons it became of interest to compare further the effects of phlorizin and dinitrophenol on mitochondrial oxidation. The data in Table V are from an experiment designed to compare the effects of phlorizin and dinitrophenol on the one step oxidation of α-ketoglutarate in the presence of malonate. The theoretical P:O ratio for this oxidation is 4. It is seen in Column 4, Table V that the P:O of 3.54 in the control came close to this theoretical figure. Furthermore, dinitrophenol (10^{-4} M) reduced the P:O to 0.48. In the presence of the dinitrophenol, although the P:O was reduced from 2.50 to 0.30, the phlorizin still produced its characteristic inhibition of oxygen utilization (from 4.20 to 3.01). Thus this experiment serves to substantiate what had been surmised, namely, that the phlorizin and dinitrophenol effects on oxidation and phosphorylation are different both in kind and site of action.

SUMMARY

Phlorizin in low concentrations can inhibit oxidation of several substrates and decrease the efficiency of oxidative phosphorylation in kidney and liver mitochondria. The oxidation-reduction state of the electron carrier coenzymes of the mitochondria can markedly alter this phlorizin effect. The adenine nucleotide reversal of the phlorizin inhibition of oxidative metabolism first noted in homogenates has been shown to occur in mitochondria as well. In addition, it has been shown that phlorizin inhibition of α-ketoglutarate oxidation is reduced by malonate. The possibility that this adenosine triphosphate and malonate "protection" is related to the protective effect of these two substances on mitochondrial structure has been discussed.

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