A STUDY OF SOME EFFECTS OF PHLORIZIN ON THE METABOLISM OF KIDNEY TISSUE IN VITRO*

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The glycoside phlorizin has been of interest ever since von Mering discovered that its administration causes a profuse glycosuria (1). The demonstration that phlorizin produces this effect by the complete inhibition of glucose reabsorption in the tubule (2, 3) has posed investigators with the problem of explaining its mechanism of action. An understanding of the nature of phlorizin inhibition of transport has wider physiological implications because it would be the key to the nature of the transport process itself.

Because the transport of substances across cells, often against osmotic and chemical gradients, requires the expenditure of energy by the cell, in any complete explanation of the mechanism of a transport inhibitor like phlorizin a study of its effect on the energy metabolism of the cell must be carried out. For this reason, the present experiments were performed, the purpose of this paper being to present some studies of the effect of phlorizin on the intermediary metabolism of kidney cortex and to discuss the results in relation to the phlorizin inhibition of transport.

Methods

Experiments were performed on homogenates of cortex of guinea pig kidney, freed from endogenous substrate by centrifugation and fortified with magnesium ions, adenosine triphosphate (ATP), and specific substrate. Studies in microrespiration were carried out in the Warburg apparatus at 37° in an atmosphere of 100 per cent oxygen, the period of incubation being 45 minutes.

After a blow on the head, the guinea pig was exsanguinated. The kidneys were quickly removed, demedullated, and weighed, and then homogenized in a Potter-Elvehjem apparatus with about 30 ml. of iced 1 per cent KCl. The homogenate was then centrifuged for 1 hour at 6000 r.p.m. in the cold in a Servall angle head centrifuge. The supernatant fluid was poured off and the tissue again suspended and homogenized in iced 0.1

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M phosphate buffer, pH 7.4, in 0.9 per cent KCl. The volume of the homogenate was adjusted to give a final tissue concentration of 100 mg. per ml., and 2 ml. were added to each flask, which had been previously chilled and charged with magnesium, ATP, substrate, and phlorizin. Ion-free water was added to give a final volume of 3 ml. Without added substrate, these homogenates had essentially no respiration as a result of the washing and centrifuging procedure; therefore, the oxygen consumptions recorded below are the result of the specific substrate added.

At the end of the 45 minute incubation period, the flasks were placed in cracked ice and 2 ml. of 25 per cent trichloroacetic acid were added quickly. Citrate was determined on the tissue filtrates by the method of Pucher, Sherman, and Vickery (4), as modified by Buffa and Peters (5), and the citrate utilized was the net difference between the amount added and the amount found at the end of 45 minutes (this quantity is referred to as $Q_{citrate}$). Q_{O_2} consists of the microliters of O_2 consumed per flask per hour, each flask containing approximately 200 mg. of tissue.

Results

Inhibition of Citrate Oxidation by Phlorizin—In Table I are presented the results of an experiment in which phlorizin, in a concentration of 5×10^{-4} M, caused a marked decrease in the oxidation of citrate. The fall in Q_{O_2} from 1050 to 405 was accompanied by a parallel drop in $Q_{citrate}$ from the control rate of 24.0 to the inhibited rate of 13.9. The relationship between concentration of phlorizin and inhibition of citrate oxidation is shown in Fig. 1. It is evident that the inhibition begins at a phlorizin concentration of about 1×10^{-4} M and becomes nearly maximal, with a 50 per cent inhibition, at a concentration of 1×10^{-3} M. These findings (Table I and Fig. 1) are in essential agreement with those reported for a rat kidney mince by Shapiro (6).

Inhibition of Oxidation of Other Substrates of Tricarboxylic Acid Cycle by Phlorizin—As an extension of these findings with citrate, it was decided to examine the effect of phlorizin, in a concentration of 5×10^{-4} M, on the oxidation of equimolar concentrations of each of the other intermediates of the tricarboxylic acid cycle. Accordingly experiments were performed which measured Q_{O_2} with the sodium salts of pyruvate, citrate, α-ketoglutarate, succinate, fumarate, L-malate, and oxalacetate, over a 30 minute period, during which time the rate of oxygen consumption was constant. 50 μmoles of substrate were used in each experiment, the results of which are presented in Table II. It is evident that phlorizin caused an inhibition in the oxidation of each of these other substrates of the tricarboxylic acid cycle. Since, in each case, the oxygen consumption was essentially zero without added substrate, it can be assumed that the oxygen consumption

values recorded here are the result of the oxidation of the specific substrate added, and therefore that the phlorizin has acted on the oxidation of that particular substrate in each separate instance. Thus the effects of phlorizin on renal metabolism, at least in the washed homogenate, appear to

TABLE I

Effect of Phlorizin on Citrate Oxidation of Washed Homogenate of Guinea Pig Kidney Cortex

Experimental condition	Citrate oxidation	
	Q_{O_2}	$Q_{citrate}$
Control	1050	24.0
Phlorizin 5×10^{-4} M	405	13.9

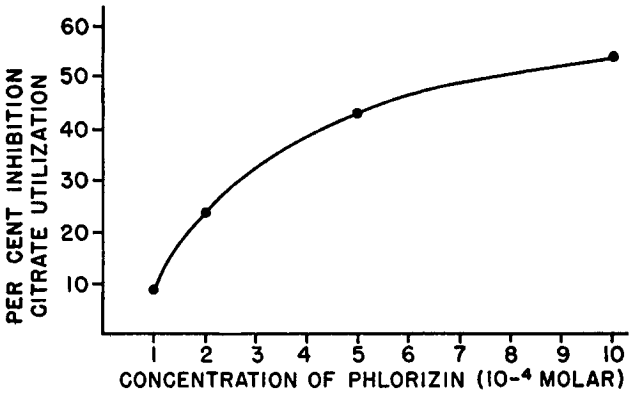

FIG. 1. The relationship between phlorizin concentration and per cent inhibition of citrate utilization by guinea pig kidney cortex. The reaction mixture consisted of $MgCl_2$ 4.1 μmoles, citrate 50 μmoles, and phlorizin, to the concentration shown, and was dissolved in hot water and neutralized. 2.0 ml. of homogenate containing 200 mg. of tissue in 0.1 M phosphate buffer, pH 7.4, containing 0.154 M KCl. Final volume, 3.0 ml.; gas phase 100 per cent oxygen.

involve some cellular site which affects the whole system of oxidative metabolism.

Effect of Other Substrates of Tricarboxylic Acid Cycle on Phlorizin Inhibition of Citrate Metabolism—In his paper, Shapiro (6) postulated that phlorizin specifically produces an inhibition in the dehydrogenation of citrate in kidney tissue with a consequent defect in the phosphorylation of creatine. He further reported that succinate oxidation was not affected by phlorizin, thus placing the phlorizin block above succinate in the chain of biological oxidations. Indeed, Shapiro found that, when suc-

cinate was added to the phlorizin-treated kidney mince, oxidative metabolism and the phosphorylation of creatine were restored to control values.

In view of the more general effects of phlorizin on oxidative metabolism observed here, it was decided to investigate further this effect of succinate and the other cycle intermediates on the phlorizin inhibition of citrate oxidation. Accordingly, the following experiments were carried out. In each experiment phlorizin concentration was 5×10^{-4} M, and 50 μmoles of citrate and 20 μmoles of the second substrate were added. The results of such experiments appear graphically in Fig. 2. In these data the control rate of citrate oxidation is given a value of 100 per cent in each experiment and is so represented in the first bar of each group. In the presence of phlorizin and the second substrate plus phlorizin, the citrate

TABLE II

Inhibition of Oxidation of Each of Substrates of Tricarboxylic Cycle by Phlorizin 5×10^{-4} M Washed Homogenate of Guinea Pig Kidney Cortex

Substrate	Oxygen consumption	
	Control	Phlorizin 5×10^{-4} M
Pyruvate	192	95
Citrate	1050	420
α-Ketoglutarate	883	589
Succinate	1555	1049
Fumarate	1480	992
L-Malate	875	604
Oxalacetate	1049	727

oxidation is represented as per cent of control rates of citrate utilization in the second and third bars, respectively. In none of the experiments did the second substrate cause a significant change in control rate of citrate oxidation in the absence of phlorizin. However, in the *presence* of phlorizin there was a dramatic effect. Rather than reversing the phlorizin inhibition of citrate oxidation, the second substrate here actually potentiated the inhibition in the cases of succinate, α-ketoglutarate, fumarate, and oxalacetate. L-Malate appeared to be without effect.

That this potentiation of the phlorizin inhibition depends on the concentration of the second substrate is illustrated in Fig. 3, in which succinate was studied. 50 μmoles of citrate were present in each flask and phlorizin concentration was kept constant at 5×10^{-4} M. As the quantity of succinate was increased in the presence of phlorizin, the inhibition of citrate oxidation became proportionately greater. This same relationship was found also when α-ketoglutarate or fumarate was the second substrate.

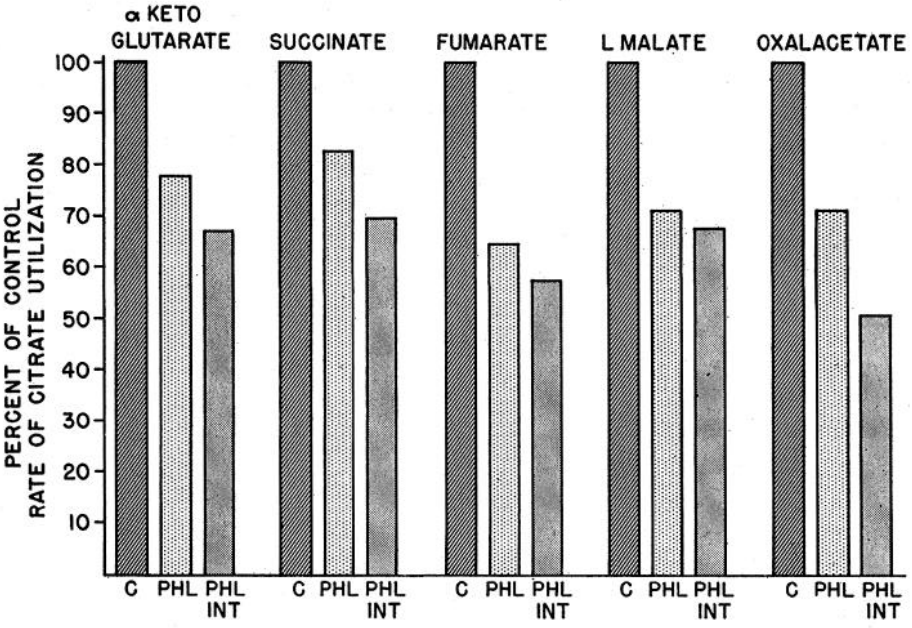

FIG. 2. Experiments illustrating the way in which a second substrate potentiates the inhibition of citrate oxidation by phlorizin. Conditions as described previously. Phlorizin 5×10^{-4} M; second substrate 20 μmoles. Phl = phlorizin; C = citrate; Int = inhibited.

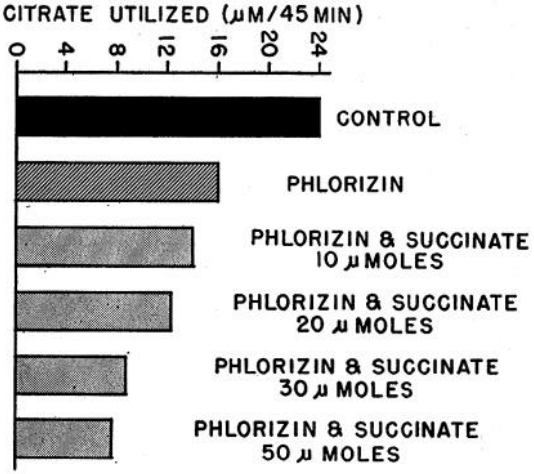

FIG. 3. Effect of succinate concentration on the inhibition of citrate oxidation by phlorizin 5×10^{-4} M. Conditions the same as previously.

Thus in the washed and fortified homogenate, succinate and several other substrates actually enhance, rather than reverse, the phlorizin inhibition of citrate oxidation. We do not at the present have an adequate explanation of this potentiating effect of the second substrate on phlorizin inhibition.

Experiments Leading to Hypothesis Concerning Site of Action of Phlorizin—The observation that phlorizin inhibits the oxidation of pyruvate and each of the intermediates of aerobic metabolism suggested two possible sites of this inhibitory action, either (1) those reactions leading from pyruvate to acetate, its activation, and condensation to citrate, or (2) the electron carrier system that transports substrate hydrogen to molecular oxygen. Each of these two possibilities was then explored.

Is Site of Action of Phlorizin on Activation of 2-Carbon Fragment and Synthesis of Citrate?—It is well known that fluoroacetate causes a profound block in oxidative metabolism (5). This inhibition is quite specific and results from the activation and conversion of fluoroacetate to fluorocitrate, which then strongly inhibits the enzyme aconitase (7). In the presence of fluoroacetate or fluorocitrate *in vitro* or *in vivo*, there is apparently no defect in 2-carbon condensation to citrate, and this substance accumulates in large amounts behind the aconitase block in poisoned tissues.

It was decided to take advantage of this "trapping" of citrate which occurs after fluoroacetate to see whether phlorizin would affect any of the ATP-requiring activation and condensation steps leading from pyruvate to a 2-carbon fragment and then to citrate synthesis. If phlorizin were to affect any of these steps, its presence in the system should alter the quantity of citrate accumulated in the fluoroacetate-poisoned tissue. Accordingly homogenates were allowed to respire in the presence of sodium fluoroacetate and phlorizin. Pyruvate and fumarate were used as substrate and "sparker." At the end of 45 minutes the reaction was stopped with trichloroacetic acid and the amount of citrate determined (Fig. 4).

The control homogenate accumulated 0.419 μmole of citrate. In the next three bars is represented the citrate which accumulated when phlorizin and fluoroacetate were added together in three different time sequence combinations; *i.e.*, preincubation of homogenate with fluoroacetate for 10 minutes, then phlorizin added from the side arm; next preincubation with phlorizin prior to fluoroacetate; and finally preincubation with both present. In all cases, as in the last three bars, the accumulation of citrate after fluoroacetate was essentially the same, and the phlorizin, no matter when added, had no effect on the rate of citrate synthesis. Thus it would appear that phlorizin does not inhibit any of the steps leading from pyruvate to acetate, and its activation and condensation to citrate.

Is Site of Action of Phlorizin in Electron Carrier System?—Any inhibi-

tion in this system, either of electron transport or coupled phosphorylation, would cause a depression of oxidative metabolism in tissues. On the hypothesis that phlorizin might inhibit the normal phosphate acceptor that functions with electron transport, preliminary studies were carried out on the inhibition of oxidation by phlorizin in the presence of increasing amounts of ATP. It was reasoned that the addition of excess ATP to the homogenate poisoned with phlorizin might bypass the block and furnish sufficient uninhibited phosphate acceptor to allow normal oxidation to proceed. These experiments revealed that such was the case. With

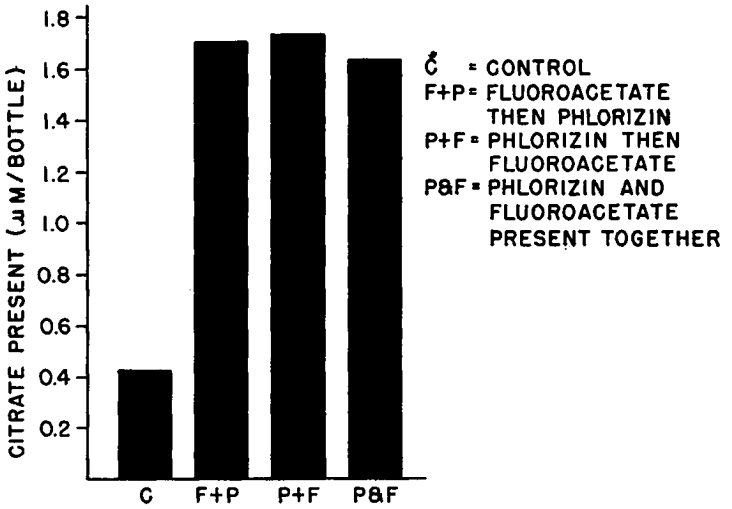

FIG. 4. The effect of phlorizin on synthesis of citrate from pyruvate and fumarate in the fluoroacetate-poisoned homogenate. The reaction mixture consisted of sodium fluoroacetate 1.4×10^{-3} M, sodium pyruvate 0.016 M, sodium fumarate 0.003 M. Other conditions as previously described.

excess ATP, phlorizin, in usually inhibitory concentrations, was now without effect on the oxidation of the intermediates of the aerobic cycle.

In an experiment illustrating this effect and its dependence on the amount of ATP added in excess (Table III), citrate was the substrate. 1.8 μmoles of ATP were found to support maximal citrate oxidation in this preparation and, at this level of ATP, phlorizin, 5×10^{-4} M, was markedly inhibitory. As the ATP was increased to 3.6 μmoles, the phlorizin was less inhibitory, and at levels of ATP of 9.0 μmoles or above phlorizin had no inhibitory effect at all.

Reversal of Phlorizin Inhibition of Citrate Oxidation by Adenine Nucleotides—The evidence now at hand indicates that adenosine diphosphate (ADP) is the more specific phosphate acceptor in maintaining the respira-

tion rate and the steady state of reduced diphosphopyridine nucleotide (DPNH) in the electron carrier chain of mitochondria (8, 9). ATP seems to serve this function only as a result of its conversion to ADP (9). Adenylic acid (AMP), on the other hand, appears to be less effective as a phosphate acceptor. Presumably its ability to support respiration depends on its ability to be phosphorylated to ADP. Therefore it was decided to compare these three adenine nucleotides with regard *first* to their relative abilities to support citrate oxidation in the washed homogenate, *second* to the capacity of phlorizin to produce its characteristic inhibition in the presence of each, and *third* to the effect of an excess of each in preventing the phlorizin inhibition. In each experiment 50 μmoles of citrate were present. Phlorizin concentration was 5×10^{-4} M and, initially, each adenine nucleotide was added in an amount of 1.8 μmoles per flask. The results of such a comparison experiment are presented in Fig. 5.

TABLE III

Reversal of Phlorizin Inhibition of Citrate Oxidation by Adenosine Triphosphate

The experiment shows effect of increasing the ATP concentration.

	Experimental condition, μmoles	Q_{O_2}	$Q_{citrate}$
Control	ATP 1.8	982	28.6
Phlorizin	" 1.8	556	18.9
"	" 3.6	807	22.2
"	" 9.0	1100	29.7
"	" 18	1121	29.3
"	" 23	1100	29.9

Control rates of citrate oxidation were comparable with the three different adenine nucleotides, and with each there was a phlorizin inhibition. In like manner an excess of each was quite effective in reversing the inhibitory effect of phlorizin.

Thus in the results of these experiments there is some correlation between the ability of an adenine nucleotide to act as a physiological phosphate acceptor (or to give rise to one) and its ability to counteract the inhibitory properties of phlorizin on oxidative metabolism. The fact that AMP supported good respiration in the control tissue and reversed the inhibitory effect of phlorizin indicates that it must have been able to give rise to adequate amounts of ADP for this purpose.

Comparison of Inhibitions of Citrate Oxidation Caused by Phlorizin and Fluoroacetate—In order to delineate further the inhibitory site of phlorizin and this adenine nucleotide reversal, and to differentiate it sharply from specific inhibitions such as malonate or fluoroacetate which occur within

the tricarboxylic acid cycle itself, the following experiment was performed. A comparison was made of the effect of ATP on the separate inhibitions

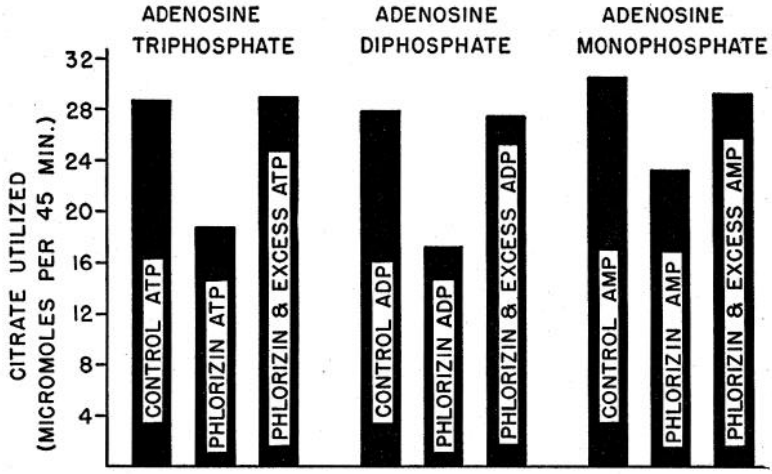

FIG. 5. The reversal of phlorizin-inhibited citrate oxidation by excess adenine nucleotides. Conditions as previously described, with special details in the text. Phlorizin concentration 5×10^{-4} M.

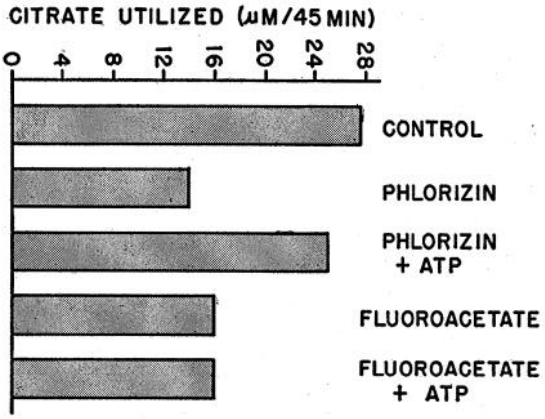

FIG. 6. Comparison of the effects of adenosine triphosphate, 9 μmoles, on the inhibitions of citrate oxidation caused by fluoroacetate and phlorizin. Conditions are as previously described, with details in the text.

of citrate oxidation produced by phlorizin and fluoroacetate, the results of which are illustrated in Fig. 6. The phlorizin, 5×10^{-4} M, and fluoroacetate (500 γ per flask) each produced almost identical inhibitions of citrate utilization, as are seen in the second and fourth bars. However, in contrast to this is the differing effect of ATP on the two inhibited sys-

tems. Excess ATP characteristically prevented the phlorizin inhibition; however, it was completely without effect on the fluoroacetate-inhibited system. Thus this experiment reinforces the conclusions drawn from the experiment of Fig. 5. Fluoroacetate, when converted to fluorocitrate, inhibits strongly and specifically at one point in the tricarboxylic acid cycle, and excess ATP neither prevents nor reverses the inhibited citrate oxidation that results. On the other hand phlorizin inhibits the oxidation of citrate in a different way, with more general effects somewhere lower down in the scheme of biological oxidations, and adenine nucleotides prevent this inhibition from occurring.

DISCUSSION

The present studies with phlorizin show that this substance, in reasonably small concentrations, has a marked effect on aerobic metabolism of kidney, with a sharp reduction in the capacity of the cells to store the energy from biological oxidations.

The evidence appears to support the concept that phlorizin somehow inhibits the capacity of ADP to accept inorganic phosphate in the coupled oxidative phosphorylations of the electron carrier system. As Chance and Williams have shown, in their excellent studies of the steady state of the components of this system (9), the addition of ADP to mitochondria containing reduced DPN stimulates oxygen consumption as reduced DPN is oxidized and hydrogen is carried on through flavin adenine nucleotides and cytochromes to molecular oxygen. When ADP is absent or when it has been quantitatively converted to ATP, oxygen consumption stops.

Since the phlorizin block does not occur in the presence of excess adenine nucleotide, it seems reasonable to speculate that in the phlorizinized tissue ADP is being inhibited in its function of accepting phosphate. The exact way in which phlorizin does this is not clear at present. However, since there is a relation between the amount of adenine nucleotide present and the degree of phlorizin inhibition, it looks as if there is a competition between phlorizin and the adenine nucleotide for the active centers of the enzyme system which transfers phosphate to the adenine nucleotide, or perhaps in the phlorizinized tissue there is formed a phosphorylated phlorizin instead of phosphorylated adenine nucleotide. The search for a substance is in progress.

It seems clear that phlorizin causes a major disruption of energy metabolism of the kidney cell *in vitro*, but it is difficult to see why alterations of such magnitude in energy metabolism should have such relatively discrete and temporary effects on tubular transport. On the side of reabsorption, it is glucose transport that is quite specifically inhibited. The energy-requiring mechanisms for the reabsorption of amino acids, acetoacetate, phosphate, and some other components of the glomerular filtrate

are unaffected by phlorizin. When compared to some other inhibitors, phlorizin is relatively non-toxic and, moreover, its effect on glucose reabsorption is quite transient, lasting only an hour or so after a single injection of a few hundred mg. in dog or man.

In spite of these difficulties of interpretation there would seem to be significance in the observations reported here. One can suppose that the energy requirements of different transport systems neither are equal to nor demand the coupling of their energy in exactly the same way. The phlorizin inhibitions noted here were not greater than 50 per cent at the highest concentrations studied. Perhaps those transport systems that are more sensitive to phlorizin are more exacting in their energy requirements.

The supposition is, of course, that phlorizin gains entrance to the cell. To have an inhibitory effect on the electron carrier system it would have to reach the intracellular mitochondria. Studies in this laboratory on the excretion of phlorizin in the dog and a few observations on the aglomerular fish *Lophius americanus* indicate that phlorizin is secreted by the tubular cells into the urine, and thus enters the tubular cell as a part of its renal excretion. It is reasonable to conclude that it is concentrated there to levels sufficient to be inhibitory to the several transport mechanisms it is known to affect.

SUMMARY

Relatively small concentrations of the glycoside phlorizin inhibit the oxidation of all of the substrates of the tricarboxylic acid cycle in washed and fortified homogenates of guinea pig kidney cortex. The inhibition, in the case of citrate, is potentiated when another cycle substrate is added. Respiration and phlorizin inhibition occur in the presence of adenosine triphosphate, adenosine diphosphate, and adenylic acid. In the presence of an excess of any of these three adenine nucleotides, the phlorizin inhibition of oxidation is either partly reversed or prevented altogether. It is theoretically concluded from these data that phlorizin interferes with the phosphate acceptor rôle of ADP in the coupled phosphorylations of the electron carrier system in kidney.

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