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The Mechanism of Phloridzin Glucosuria

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Glucose is reabsorbed in the kidney by the proximal tubule (Walker & Hudson, 1937). Lambrechts (1937) has shown that phloridzin is concentrated in the tubule at the same site. Although the *locus* of the action of phloridzin in producing glucosuria is thus identified as the proximal tubule, none of the theories which have been put forward explains to entire satisfaction the mechanism of the reabsorption of glucose and the manner of the action of phloridzin on this process. It is generally assumed that glucose is actively reabsorbed in the kidney by a process of phosphorylation. It has been suggested that phloridzin prevents glucose reabsorption by inhibiting glucose phosphorylation (Lundsgaard, 1933; Kalckar, 1936). This view is based mainly on the demonstration that phloridzin can inhibit glucose phosphorylation *in vitro*, e.g. by kidney slices, mince or extracts. The concentration of phloridzin necessary to inhibit phosphorylation of sugar is, however, definitely higher than the minimum concentration which brings about complete poisoning of reabsorption in the kidney (Lundsgaard, 1935). Kidneys of animals poisoned with phloridzin phosphorylated glucose at the same rate as normal kidneys.

Phloridzin exerts inhibiting effects on a wide variety of enzyme systems. Since such effects had not been observed except at a relatively high concentration of phloridzin, it was considered that they are irrelevant to the role of phloridzin in glucosuria. Re-examination of the validity of this view appeared desirable.

In a systematic test of the action of phloridzin on the different enzymes of carbohydrate desmolyse (Shapiro, 1939), the dehydrogenases of citric and pyruvic acid and certain systems which, by coupling with these dehydrogenases, generate high energy phosphate bonds, have been found to be highly susceptible to phloridzin inhibition. Marked inhibition of the susceptible systems was observed even at concentrations of phloridzin (0.0002–0.001 M) which are too low to affect glucose phosphorylation. It seems probable, therefore, that inhibition of glucose phosphorylation by phloridzin is but one of several secondary results of the action of phloridzin on reactions which generate high-energy phosphate bonds. The present paper summarizes data in proof of this statement. Experiments are also reported to show that the inhibitory effect of phloridzin on high-energy phosphate bond formation is manifested in the kidney of phloridzinized rats *in vivo* as well as *in vitro*.

METHODS

Rat kidneys were cooled on ice, the cortex separated and made into a fine mince with a scissors. When not otherwise stated, the mince (0.3–1.0 g.) was suspended in 5 vol. of Krebs's phosphate- or bicarbonate-Ringer solution (Krebs, 1933). Phloridzin (0.02 M) was dissolved in hot water and added to the reaction flasks to obtain the desired final phloridzin concentration.

Glucose was estimated according to Somogyi (1937). Lactic acid was determined by a modification of Friedemann's method according to Lieb & Zacherl (1932), inorganic phosphate according to Lohmann & Jendrassik (1926), creatine-phosphate as described by Potter (1945),

pyruvic acid according to Clift & Cook (1932) and citric acid according to Pucher, Sherman & Vickery (1936).

The tables contain representative results.

RESULTS

Inhibition of glucolysis by phloridzin

When incubated with kidney mince in phosphate-Ringer solution, glucose was utilized under aerobic as well as anaerobic conditions. Phloridzin at a concentration of 0.001 M inhibited glucose utilization in aerobic conditions but was relatively ineffective in the anaerobic experiments (Table 1). Concentrations of phloridzin below 0.001 M had no regular inhibitory effect on glucose utilization. A similar finding was indicated by analyses in which lactic acid formation was measured (Table 2).

Table 1. *Inhibition by phloridzin of glucose utilization in kidney mince*

1 g. kidney mince suspended in 5 ml. phosphate-Ringer solution containing 0.1 % glucose, incubated for 1 hr. at 37°.

Exp. no.	Gas	mg. glucose utilized at phloridzin conc.		Inhibition (%)
		0	0.001 M	
1	Air	0.96	0.36	63
	Nitrogen	1.08	0.96	12
2	Air	0.81	0.48	44
	Nitrogen	1.00	0.96	4
3	Air	0.87	0.51	37
	Nitrogen	0.90	0.81	10

Table 2. *Inhibition by phloridzin of lactic acid formation in kidney mince*

Same mixture as above but with 0.2 % glucose, incubated for 3 hr.

Exp. no.	Gas	mg. lactic acid formed at phloridzin conc.		Inhibition (%)
		0	0.001 M	
4	Air	1.38	0.94	32
	Nitrogen	1.72	1.58	8
5	Air	1.56	0.80	48
	Nitrogen	1.46	1.44	0
6	Air	2.20	1.24	43
	Nitrogen	1.72	1.60	7

Kidney mince was made weak in phosphate by dilution with bicarbonate-Ringer solution, 1:10 and 1:15. Phloridzin (0.001 M) failed to affect glucolysis by the diluted preparation (Table 3). Phloridzin inhibition reappeared, however, following addition of phosphate to the diluted preparation. When kidney mince was diluted with phosphate-Ringer solution no diminution in glucolysis susceptibility to 0.001 M-phloridzin occurred.

Table 3. *Dependence of the inhibition of glucose utilization on phosphate*

Dilution of kidney mince	Inhibition (%) by 0.001 M-phloridzin	
	In bicarbonate-Ringer solution	In phosphate-Ringer solution
1 g. in 5 ml.	41	41
1 g. in 5 ml.	28	37
1 g. in 10 ml.	11	36
1 g. in 10 ml.	0	27
1 g. in 15 ml.	5	30
1 g. in 15 ml.	0	28
1 g. in 15 ml. + 1 ml. phosphate-Ringer solution	18	—
1 g. in 15 ml.	0	—
1 g. in 15 ml. + 1 ml. phosphate-Ringer solution	27	—

Inhibition of dehydrogenase systems by phloridzin

The dependence of glucolysis inhibition by phloridzin on presence of oxygen and inorganic phosphate suggests that the point of attack of phloridzin is an oxidative process with which phosphorylation is coupled. In agreement with other authors it was found that phloridzin does not markedly reduce the respiration rate of the kidney. A number of individual respiratory systems was tested and several were found to be highly susceptible to phloridzin poisoning. The most marked inhibition was found in respect of the dehydrogenases of pyruvic and citric acids. These two dehydrogenases were completely inhibited by 0.001 M-phloridzin: in presence of phloridzin, addition of pyruvic and citric acid did not accelerate the decolorization of methylene blue.

Table 4. *Inhibition of dehydrogenases by phloridzin*

Kidney cortex mince, washed four times with M/15-phosphate buffer pH 5, suspended in 4 volumes of phosphate buffer at pH 7.4. 1 ml. of the suspension was introduced into a Thunberg tube and 0.1 ml. methylene blue (0.1 %) and 0.2 ml. substrate (M/20) were added. The tubes were evacuated and incubated at 37°.

Exp. no.	Substrate	Time of decolorization (min.)			
		Without phloridzin		With 0.001 M-phloridzin	
		No substrate	With substrate	No substrate	With substrate
1	Pyruvate	70	40	90	90
2	"	40	15	55	55
3	"	90	55	140	135
4	Citrate	41	14	75	56
5	"	90	37	97	75
6	"	90	27	140	135
7	Succinate	45	16	63	22
8	"	41	6	75	6
9	"	90	20	140	26

Succinic dehydrogenase, on the other hand, was not inhibited by the same concentration of phloridzin (Table 4).

Utilization of pyruvic acid and citric acid by kidney mince was found to be partially inhibited by 0.001M-phloridzin. This suggests that not all the pathways of dissimulation are poisoned by phloridzin. Inhibition of pyruvic and citric acid utilization by phloridzin was nevertheless measurable even at concentrations of phloridzin as low as 0.0002M (Table 5).

Table 5. *Inhibition by phloridzin of pyruvate utilization in kidney mince*

1 g. kidney mince incubated in 5 ml. phosphate-Ringer solution containing 0.1% sodium pyruvate for 2 hr. at 37°.

	Pyruvate utilized (mg.)			
	Without phloridzin	0.001M-phloridzin	0.0002M-phloridzin	0.0001M-phloridzin
1	2.23	1.44	1.96	2.07
2	2.04	1.62	1.82	2.00
3	1.55	1.00	1.23	1.59

Table 6. *Inhibition by phloridzin of citrate utilization in kidney mince*

As in Table 5, but citrate (0.1%, Na salt) instead of pyruvate.

	Citrate utilized (mg.)			
	Without phloridzin	0.001M-phloridzin	0.0002M-phloridzin	0.0001M-phloridzin
1	1.45	1.05	1.12	1.34
2	1.50	0.90	1.08	1.39

Table 7. *Inhibition of creatine-phosphate synthesis by phloridzin*

0.5 g. kidney mince suspended in 1.5 ml. of a mixture containing: 100 mg. creatine + 4 ml. water + 1 ml. KCl (0.5M) + 1 ml. MgCl₂ (0.1M) + 1 ml. NaF (0.25M) + 1 ml. sodium phosphate buffer (0.2M, pH 7.6), incubated for 20 min.

	Creatine-phosphate-P (μg.) synthesized with phloridzin						
	0	M/500	M/1000	M/1500	M/3000	M/4000	M/10000
1	35	4	9	11	14	18	32
2	40	5	5	9	—	12	22
3	38	—	3	—	6	8	—

Inhibition of coupled phosphorylation by phloridzin

As phosphorylation of adenylic acid is known to be coupled with pyruvate and citrate oxidation (Ochoa, 1941; Colowick, Welch & Cori, 1940), it is reasonable to consider that the inhibition of gluco-lysis and of glucose reabsorption in the kidney by phloridzin may be secondary to the primary effect of phloridzin on a susceptible dehydrogenase. Experiments were designed to study the influence of phloridzin on the phosphorylation of adenylic acid

in the kidney. In the presence of phloridzin, permanent inhibition of phosphorylation was observed. The evidence of this finding was, however, inconclusive, as even in absence of phloridzin very low activity was found. To increase the rate of phosphorylation, a phosphate acceptor (creatine) was added to the kidney mince and high concentrations of fluoride and Mg⁺⁺ were used to inhibit adenosine-pyrophosphatase (Potter, 1945).

Phosphorylation of creatine in kidney mince was completely abolished by 0.001M and was markedly retarded by concentrations down to M/4000 phloridzin (Table 7). Small amounts of succinate (M/800) increased creatine phosphorylation without effecting phloridzin inhibition; concentrations of succinate of the order of 0.01M, on the other hand, completely abolished phloridzin inhibition (Table 8).

Table 8. *Influence of succinate addition on the phloridzin inhibition of creatine-phosphate synthesis*

Conditions, other than succinate concentration, same as for Table 7.

Addition	Creatine-phosphate-P (μg.) synthesized	
	Without phloridzin	M/1000 phloridzin
No addition	23	6
M/800 succinate	54	10
M/100 succinate	59	57
No addition	28	5
M/800 succinate	43	8
M/100 succinate	41	37

Inhibition of coupled phosphorylation by injected phloridzin

Phloridzin, dissolved in propylene glycol, was injected into rats in a dosage of 25 mg./100 g. body weight. Control rats were injected with propylene glycol only. After half an hour, a second injection was given. The animals were sacrificed after an additional 15 min. Creatine-phosphate synthesis was then examined as before in the kidney tissue of these animals, M/800 succinate being added in order to raise the phosphorylation figures. The experimental and control groups numbered ten rats each, the other conditions were the same as in Table 7. 32 ± 2 μg. creatine-phosphate-P were synthesized by the control; 17 ± 3 μg. creatine-phosphate-P by the phloridzin injected group. It follows that a significant inhibition of the creatine-phosphate synthesis is caused by injection of phloridzin *in vivo*.

DISCUSSION

It has been shown in the present paper that pyruvic and citric acid oxidation in kidney is inhibited by a concentration of phloridzin as low as 0.0002M. The

same phloridzin concentration also inhibits processes which generate high-energy phosphate bonds, and which are known to be coupled with these oxidations (Ochoa, 1941; Colowick *et al.* 1940). It seems necessary to assume that inhibition of susceptible dehydrogenase systems is the primary basis of phloridzin action in the kidney. This assumption is corroborated by the fact that phloridzin administered *in vivo* by injection is retained by the kidney in sufficient concentration to cause marked inhibition of creatine phosphorylation by the kidney. Lundsgaard (1935) showed in a 'pump-lung-kidney' preparation of a dog that complete poisoning of glucose reabsorption is ensured by 0.5–1.0 mg. of phloridzin/g. kidney. This dosage level corresponds to a concentration of $m/3000$ – 5000 in our test mixtures (1 g. kidney in 5 ml.). In this range of phloridzin concentration creatine phosphorylation is almost entirely inhibited. Glucose utilization as well as glucose phosphorylation (Kalckar, 1936) are inhibited to a similar degree only by 5–10 times as much phloridzin. Action of phloridzin on glucose utilization and phosphorylation may therefore be regarded as secondary to the inhibition by phloridzin of the production of high energy phosphate bonds.

These results are in agreement with the finding of Rapoport, Nelson, Guest & Mirsky (1941) who showed with the aid of labelled phosphate that the turnover of labile organic phosphate is reduced by phloridzin injection. Similar results were obtained by Kaplan & Greenberg (1944) with liver tissue. Our data suggest that the primary inhibitory action of phloridzin is exerted neither on phosphate transfer to adenylic acid nor on glucose phosphorylation by adenosine-pyrophosphate, as suggested by Beck (1942), but on oxidations with which phosphorylation of adenylic acid is coupled. This point is substantiated by the finding that the synthesis of high energy phosphate bonds proceeds normally in

the presence of phloridzin if a substrate such as succinate, whose oxidation is not inhibited by phloridzin, is added to the system.

The assumption that phloridzin inactivates systems generating high energy phosphate bonds affords a plausible explanation of the finding that inhibition of reabsorption by phloridzin is not specifically limited to the case of glucose. Ellinger & Lambrechts (1937) observed failure of reabsorption of various dyes from the tubules of phloridzinized frogs. White (1940) has shown that secretion of diodrast is diminished by phloridzin. Evidently high energy phosphate bonds are important in kidney function for other purposes besides glucose reabsorption.

SUMMARY

1. Phloridzin at a concentration of $0.001M$ inhibits glucose utilization and lactic acid formation by minced kidney cortex.

2. This inhibition is dependent upon the presence of both oxygen and inorganic phosphate.

3. Oxidation of pyruvic and citric acids, but not of succinate, is inhibited by concentrations of phloridzin as low as $0.0002M$.

4. The phosphorylation of creatine is inhibited by $m/4000$ phloridzin. The inhibition is abolished by $m/100$ succinate, but not by $m/800$ succinate which stimulates the phosphorylation in the absence of phloridzin.

5. Phloridzin given *in vivo* depresses creatine phosphorylation in minced kidney.

6. The results are explained by the assumption that phloridzin acts primarily on dehydrogenase systems that are coupled with phosphorylation. The bearing of these results on the question of the mechanism of phloridzin glucosuria is discussed.

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