CI. THE EFFECT OF PHLORIDZIN ON CARBOHYDRATE METABOLISM *IN VITRO*

BY STEFAN JOSEPH BACH

From the Biochemical Laboratory, Cambridge

(*Received 28 February 1939*)

THE metabolic effect of phloridzin has been interpreted in several different ways. Some authors have claimed a solely renal action [Erlandsen, 1910; Allen, 1913] inasmuch as phloridzin affected the permeability of the renal tubules for glucose and other substances [Underhill, 1912], thus inhibiting their reabsorption by the kidney [Verzár & Laszt, 1934]. This phenomenon was explained by the further observation of the inhibiting effect of phloridzin, both on the phosphorylation of glycogen [Wilbrandt & Laszt, 1933] and on the glycolysis in muscle and yeast (also a phosphorylating mechanism [Lundsgaard, 1933]). As this inhibiting effect was also observed on glycolysis in brain, which is generally considered to be of a non-phosphorylating nature [Ashford & Holmes, 1929], there may be reason to suggest that phloridzin, besides inhibiting phosphorylation, may exert still another metabolic effect. Underhill [1912] postulated a twofold action: (1) on the permeability of kidney tissue and (2) "upon other structures, resulting in the production of sugar". It was the possibility of this second action of phloridzin on the production of sugar which presented the problem for this work. As biological material, liver of starved rats, in which carbohydrate synthesis has previously been demonstrated in experiments *in vitro* [Bach & Holmes, 1937] was chosen. Experiments on the effect of phloridzin and other glycolytic inhibitors on such carbohydrate production, which revealed a special behaviour of phloridzin, led to further investigations.

Methods

Animals. Young rats were used, chiefly albinos, all males with an average weight of 200 g. They were always starved for 20–24 hr. before the experiments, in order to decrease the carbohydrate content of the liver. Before starving, the animals were kept on the ordinary stock laboratory diet.

Slice technique. This was the same as described previously [Bach and Holmes, 1937]. The weight of tissue was 100 mg. wet weight in all manometric experiments and varied from 300 to 500 mg. in experiments where the carbohydrate content was determined.

The slices were placed into Barcroft cups containing 3 ml. $NaHCO_3$-Ringer of *p*H 7·3 which included the substrates after adjustment to the same *p*H. The mixtures were equilibrated with a gas mixture containing 95 % O_2 and 5 % CO_2 in the aerobic, and with a mixture (passed over hot Cu filings) of 95 % N_2 and 5 % CO_2 in the anaerobic experiments.

In the experiments in which determinations of O_2, CO_2 and carbohydrate were carried out at different intervals, the time at which the vessels were placed in the water bath for incubation (before equilibration) was taken as zero.

Manometric experiments. The O_2 uptake was determined in phosphate buffer, *p*H 7·3, with Barcroft manometers containing air and with 10 % KOH in the

centre pot of the vessels. For the determination of the R.Q. the method of Dixon & Keilin [1933] was employed, and was checked in one case by the Warburg two-vessel method. The latter experiment in which glycolysis, separately determined, was allowed for was carried out with the kind help of Dr H. Laser according to Laser & Rothschild [1939].

Determination of total fermentable carbohydrate. In most experiments the procedure previously described [Bach & Holmes, 1937] was closely followed and the final sugar estimations were performed by the method of Hadedorn & Jensen [1923]. In some of the "timing" experiments in absence of phloridzin (Figs. 3 and 5) the quicker, colorimetric method of Dische & Popper [1926] was used. Blank experiments showed that the two methods agreed within 5 %. For the experiments with phloridzin the Hagedorn & Jensen method was found to be the more reliable.

Inorganic phosphate was estimated according to the method of Fiske & Subbarrow [1925] and lactic acid by the method of Friedemann *et al.* [1927].

Reagents. Phloretin was prepared in the following way: to 2 g. phloridzin suspended in 20 ml. boiling H_2O, 10 ml. 20 % H_2SO_4 were added. The mixture was heated for 90 min. on the boiling water bath. The precipitate was filtered and thoroughly washed with cold water, dried and recrystallized from abs. alcohol. 90 % yield of carbohydrate-free phloretin was obtained. M.P. 253–255°.

The other reagents including phloridzin and insulin were supplied by British Drug Houses Ltd. The potency of the insulin hydrochloride was close to 20 units per mg.

EXPERIMENTAL

Liver slices were incubated in presence and absence of NaF, iodoacetate and phloridzin. The carbohydrate content of the tissue suspension was estimated before and after the period of incubation. Results from Table I show an increase

Table I. *The effect of phloridzin, NaF and iodoacetate on carbohydrate synthesis*

Quantities: mg. fermentable carbohydrate per g. fresh liver. Experimental period 2 hr.

Initial	Final (no addition)	Increase without addition	Extra increase with added NaF 0·01 *M*	Iodoacetate 0·01 *M*	Phloridzin 0·01 *M*
2·93	5·77	+2·84	−0·37	−1·00	+2·26
—	5·23	—	−0·60	−0·51	—
2·92	4·39	+1·47	−1·04	−0·46	—
4·72	5·94	+1·22	—	—	+0·71
2·09	3·40	+1·31	—	—	+0·71
4·33	5·34	+1·01	—	—	+0·97*
7·30	7·50	+0·20	—	—	+1·40†

* 0·02*M* Phloridzin. † 0·005*M* Phloridzin.

in fermentable carbohydrate content in absence of substrates. This synthesis of carbohydrate was partly suppressed in presence of NaF and iodoacetate and was increased in presence of phloridzin. Blank experiments were carried out for all the substances added and special care was taken to allow for the possibility of the carbohydrate constituent of phloridzin being split off during the proceedings necessary for the determination of carbohydrate.

Since the substances added in these experiments are known to exert an inhibiting effect on a possible phosphorylating glycolysis, changes in the inorganic P content were expected during the period of incubation. Results in Table II however revealed a marked breakdown of organic P. The effect was of about the same order under both aerobic and anaerobic conditions and phloridzin

Table II. *Increase in inorganic phosphate content during incubation of liver slices*

Quantities: mg. inorganic phosphate per g. liver. Experimental period 2 hr. Concentration of added substances: phloridzin, 0·01 *M*; NaF, 0·01 *M*; glycogen, 2 %.

Substances added other than phloridzin	Initial	Increase in inorganic phosphate: Without phloridzin	Increase in inorganic phosphate: With phloridzin	Phloridzin effect
		Aerobic experiments		
—	0·47	+0·17	+0·14	−0·03
—	0·59	+0·27	+0·18	−0·09
		Anaerobic experiments		
—	0·47	+0·19	+0·15	−0·04
—	0·59	+0·53	+0·40	−0·13
NaF	0·67	+0·35	+0·35	0
NaF + glycogen	0·67	+0·30	+0·26	−0·04

only slightly diminished this breakdown of organic P. NaF caused no significant effect, while the small phloridzin effect disappeared when NaF was added simultaneously. Finally glycogen was added to the NaF-poisoned tissue for the purpose of enriching it in glycolysing material. The phosphate breakdown was only slightly diminished by the presence of glycogen and a little further reduced when phloridzin was added in addition.

Table III shows results of lactic acid estimations carried out in a number of experiments before and after the incubation of liver slices. Here again no consistent nor significant changes in either direction in the lactic acid content were

Table III. *Changes in lactic acid content during incubation of liver slices*

Quantities: mg. lactic acid per g. liver. Experimental period 2 hr.

	Initial	Final	Change	Substances added
Aerobic	3·46	3·75	+0·29	—
,,	3·70	3·62	−0·08	—
,,	2·31	2·69	+0·38	—
,,	3·41	3·20	−0·21	—
,,	3·41	3·52	+0·11	Insulin 0·15 mg./ml.
Anaerobic	3·40	4·05	+0·65	—
,,	3·40	3·76	+0·36	Phloridzin 0·01 *M*

observed when starved animals were used. A slight diminishing effect of phloridzin on glycolysis appeared under anaerobic conditions. Under aerobic conditions part of the lactic acid may have been oxidized, thus masking a possible formation of lactic acid. But the values obtained under anaerobic conditions were found to be of about the same order.

Finally experiments under anaerobic conditions, which eliminate both carbohydrate synthesis and carbohydrate oxidation, showed no significant changes in the carbohydrate content of the liver slices before and after the period of incubation (Table IV).

Summarizing the results of Tables II, III and IV there appears to be little evidence for the assumption that the increase in carbohydrate synthesis caused by phloridzin (Table I) may be due to an inhibition of the glycolytic disappearance of carbohydrate.

A possible effect of phloridzin on the oxidative disappearance of carbohydrate was investigated next. Figs. 1 and 2 show the rate of oxidation in liver tissue

Table IV. *Changes in carbohydrate content during incubation of liver slices under anaerobic conditions*

Quantities: mg. fermentable carbohydrate per g. liver. Experimental period 2 hr.

Initial	Final	Change	Substances added
10·86	10·00	−0·86	—
11·50	11·85	+0·35	—
4·61	4·70	+0·09	—
2·34	2·35	+0·01	Pyruvate 0·4 %
4·67	5·17	+0·50	Lactate 0·4 %
4·67	4·47	−0·20	Phloridzin 0·01 *M*

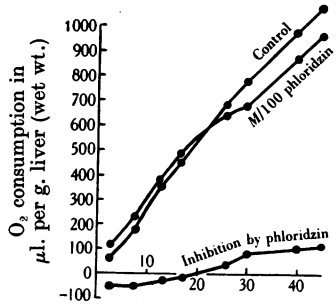

Fig. 1.

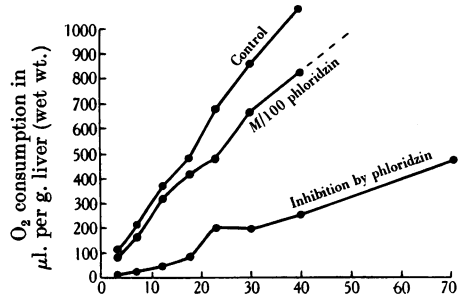

Fig. 2.

Figs. 1 and 2. The effect of phloridzin on the O_2 consumption of liver slices. 0·1 g. tissue (wet wt.) in 3 ml. phosphate buffer, *p*H 7·3 in air at 37°.

from starved rats with and without added phloridzin. It will be seen that oxidation is slightly inhibited by phloridzin, the inhibition being greatest between 20 and 30 min. This phenomenon was observed in several successive cases. This inhibiting effect of phloridzin on the tissue oxidation was more understandable when the course of carbohydrate synthesis was followed by estimating the carbohydrate content of the slices at several intervals in the experimental period (Fig. 3). In all experiments a marked decrease of carbohydrate content was observed between 20 and 30 min., reaching values even below initial carbohydrate content. This phenomenon may be explained by the assumption that the rate of synthesis during the period mentioned was slower than that of carbohydrate consumption. And as, for reasons stated above, the possibility of a mainly glycolytic disappearance of carbohydrate can be disposed of, one may be justified in correlating the phenomenon observed in Figs. 1 and 2 with that of Fig. 3. It will be seen that the critical time of carbohydrate disappearance in absence of phloridzin is almost identical with that of the greatest inhibition of O_2 consumption in presence of phloridzin. This may be a mere fortuitous coincidence, but one is tempted to connect the two results by arguing that phloridzin under the experimental conditions may inhibit an oxidative disappearance of carbohydrate in the critical period of 20–30 min. For, if the decrease in carbohydrate content was due to oxidation, and further if such oxidation was inhibited by phloridzin, a depression in O_2 consumption in that critical period would be expected. The regularity of such depression of O_2 uptake, as observed in Figs. 1 and 2, is shown in Table V in which the inhibiting effect of phloridzin on the O_2 consumption during the period of 15–30 min. was calculated for three experiments, showing an average inhibition of 120 μl. O_2 per g. liver.

Table V. *O_2 consumption from 15 to 30 min. during incubation of liver slices with and without added phloridzin*

Quantities: μl. O_2 per g. liver.

In absence of phloridzin			In presence of phloridzin			
After 15 min.	After 30 min.	During the interval of 15–30 min.	After 15 min.	After 30 min.	During the interval of 15–30 min.	Inhibition by phloridzin
352	773	421	375	683	308	113
497	862	365	412	667	255	110
530	883	353	310	526	216	137
					Average inhibition between 15 and 30 min.	120

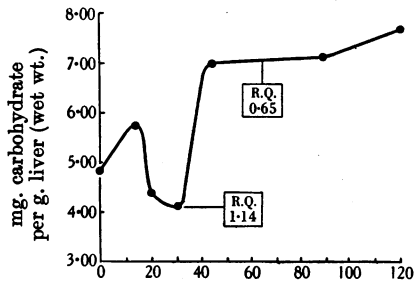

Fig. 3.

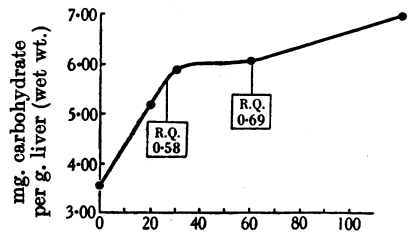

Fig. 4.

Fig. 3. The rate of carbohydrate synthesis in liver slices in absence of added substrate. 0·3–0·4 g. tissue in 3 ml. bicarbonate Ringer, *p*H 7·3 at 37°. No phloridzin.

Fig. 4. The effect of phloridzin on carbohydrate synthesis in liver slices. 0·5–0·35 g. tissue in 3 ml. phloridzin bicarbonate Ringer, *p*H 7·3 at 37°. Phloridzin concentration *M*/200.

Further evidence for this concept is given in Fig. 4, which shows the course of carbohydrate synthesis in presence of phloridzin. It will be seen that no decrease in carbohydrate content was observed during the critical period of 20–30 min. This was to be expected if phloridzin inhibited carbohydrate oxidation, thus preventing the carbohydrate content from decreasing in the period mentioned.

Experiments with insulin which according to previous findings partly inhibits the mechanism of synthesis of carbohydrate [Bach & Holmes, 1937] dispose of the idea that the disappearance of carbohydrate in the early period of incubation may be mainly due to an inhibition of carbohydrate synthesis in that period. Fig. 5 shows no significant effect of insulin on the O_2 consumption and from Fig. 6 it can be seen that the same decrease in carbohydrate content occurs at the critical time in presence of insulin, as has already been demonstrated in its absence (Fig. 3).

Finally determinations of the R.Q. carried out at two different periods of incubation in absence and presence of phloridzin supported the views stated above. If in the absence of phloridzin carbohydrate combustion was predominant in the early stage of incubation, the R.Q. for that period would be expected to approach 1. On the other hand a lower R.Q. should appear in presence of phloridzin, if carbohydrate combustion were partly suppressed. From Figs. 3 and 4 it can be seen that this is actually the case. The R.Q. in the critical time of the early stage of incubation was found to be 1·14 in absence of phloridzin and 0·58 in presence of phloridzin. For the later stage of incubation after 60 min., which

seems to be comparatively unaffected by phloridzin, the respective values were found to be 0·65 and 0·69. The latter phenomenon can be foreseen if carbohydrate oxidation takes place more rapidly in the early stage of incubation, an assumption supported by the results of Fig. 3, and further if, in accordance with the view stated above, it is this oxidation which is mainly affected by phloridzin.

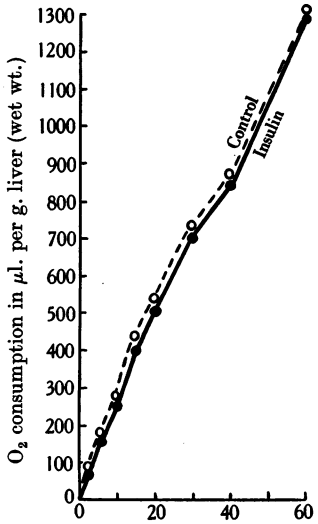

Fig. 5.

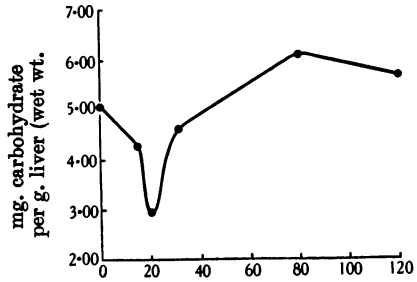

Fig. 6.

Fig. 5. The effect of insulin on the respiration of liver slices. 0·1 g. tissue in 3 ml. phosphate buffer, *p*H 7·3 in air at 37°.

Fig. 6. The rate of carbohydrate synthesis in liver slices in presence of insulin. 0·3–0·4 g. tissue in 3 ml. insulin bicarbonate Ringer, *p*H 7·3 at 37°. Insulin concentration 15 mg./100 ml.

Further it had to be considered whether phloridzin, being a glucoside, may be hydrolysed during incubation, thus increasing the carbohydrate content of the tissue suspension. This possibility could be excluded by two different types of experiments. Firstly slices were incubated in presence of phloridzin under anaerobic conditions, when both carbohydrate synthesis and carbohydrate oxidation would be eliminated, whereas a possible hydrolysis of phloridzin could still take place. As shown in Table IV, however, the carbohydrate content remained practically unchanged, indicating absence of hydrolysis. Secondly, phloretin was prepared from phloridzin by acid hydrolysis as described earlier. Care was taken to remove the glucose component completely, 97 % of the theoretical amount of which was determined in the hydrolysate. Unlike phloridzin the O_2 consumption of liver slices was inhibited by 52 % at a concentration of 0·01 *M* of the phloretin suspension, the comparative insolubility of the substance serving probably as limiting factor for its inhibitory effect. This was shown by the fact that inhibition was the same in all concentrations down to 0·002 *M* (Fig. 7). At 0·001 *M* the inhibition of O_2 consumption was still 30 %, whereas carbohydrate synthesis was hardly at all affected at that concentration, as compared with 49 % inhibition of synthesis at 0·01 *M* (Table VI). In an attempt to imitate conditions prevailing in the case of phloridzin, glucose was added to the phloretin-Ringer

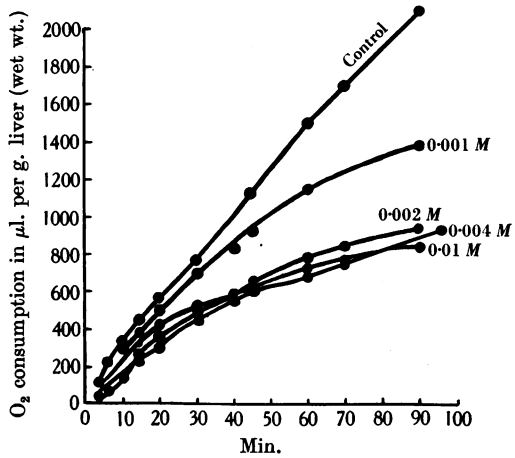

Fig. 7. The effect of phloretin on the respiration of liver slices. 0·1 g. tissue in 3 ml. of phoretin phosphate buffer, *p*H 7·3 in air at 37°.

Table VI. *The effect of phloretin on O_2 consumption and carbohydrate synthesis in liver slices*

Experimental period 2 hr.

Phloretin concentration *M*	O_2 consumption in μl. per hr. and g. liver (wet wt.)			Carbohydrate synthesis (mg. fermentable carbohydrate per g. liver)		
	Without phloretin	With phloretin	Inhibition %	Without phloretin	With phloretin	Inhibition %
0·01	1505	730	52	2·33	1·20	49
0·004	1590	745	53	—	—	—
0·002	1580	790	50	—	—	—
0·001	1620	1140	29·5	2·34	2·19	6

Table VII. *The effect of glucose in presence of phloretin on carbohydrate synthesis in liver slices*

Quantities: mg. fermentable carbohydrate per g. liver. Experimental period 2 hr.

Substances added	Before incubation	After incubation	Increase	Glucose effect in presence of phloretin
None	1·60	3·93	2·33	
Glucose 0·2 %	6·25	8·04	1·79	
Phloretin 0·001 *M*	1·60	3·29	1·69	+0·46
Phloretin and glucose	6·51	8·66	2·15	
None	3·20	5·53	2·33	
Phloretin 0·001 *M*	3·20	5·12	1·92	+1·26
Phloretin and glucose	6·96	10·14	3·18	

(0·001 *M*). It will be seen from Table VII that phloretin in presence of added glucose increased the total carbohydrate content of the liver slices by an amount exceeding the carbohydrate synthesis shown by the controls. This seems to indicate that the phloridzin effect is due to the complete glucoside rather than to either of its constituents. This experiment also confirms the view that the increase of carbohydrate content resulting from incubation of liver slices with phloridzin is not due to the liberation of the glucose component of the glucoside.

Discussion

The impairing effect of phloridzin on the mechanism concerned with the removal of carbohydrate from the blood has been recognized for a long time [Goldstein *et al.* 1932]. The discovery of its inhibitory effect on phosphorylating carbohydrate fermentation as described earlier stimulated research in that particular direction. But when the carbohydrate-sparing effect of phloridzin was observed in tissues which either, as in the case of liver, show little glycolytic activity or, as in the case of brain, are known to catalyse non-phosphorylating glycolysis [Ashford & Holmes, 1929], other possibilities had to be considered. In the particular case of this work the increased synthesis of fermentable carbohydrate in liver slices observed in presence of phloridzin was interpreted, in agreement with the views stated earlier, as an inhibited removal of carbohydrate. The observation of only small changes in inorganic phosphate content in presence and absence of glycolytic inhibitors may not alone be sufficient evidence against the assumption of a glycolytic mechanism, possibly inhibited by phloridzin, although the absence of such a mechanism has been reported by several other workers [Rona *et al.* 1925; Dann & Quastel, 1928; Tanko, 1931]. On the other hand the considerable breakdown of organic phosphate found in accordance with Rona *et al.* [1925] and Toerell & Norberg [1932] may have masked any possible rephosphorylation. But the fact that no lactic acid formation was found to take place during the incubation of liver slices (in accordance with Haarmann & Brink [1935] and Willstätter [1936]) and further, the fact that the carbohydrate level remained unchanged under anaerobic conditions, supplies additional evidence that the disappearance of carbohydrate observed in absence and inhibited in presence of phloridzin could not be of fermentative nature.

Now it is known from experiments *in vivo* that though hepatic glycogen is greatly diminished following phloridzin administration, the glucaemic level remains normal or even shows a marked increase [Schwarz & Sassler, 1928; Rathery *et al.* 1930]. The two phenomena may be the result of two separate mechanisms but the accumulation of glucose, i.e. of a readily oxidizable substance in presence of phloridzin, suggests a disturbance of the oxidative function of the liver [Nash & Benedict, 1923]. The experiments *in vitro* on the isolated organ described in this work may supply satisfactory confirmation of this view, suggested hitherto by experiments on the whole animal. The period of inhibition of O_2 consumption by phloridzin coincides well with the period of carbohydrate disappearance in its absence, thus explaining the carbohydrate-saving effect. Fleischmann [1937] observed a similar type of O_2 inhibition with phloridzin in brain slices, but failed to do so in liver or kidney slices. This result may have technical causes since he also failed to observe the familiar extra O_2 uptake after adding glucose to these organs.

Estimations of the respiratory quotient fitted in well with the views stated above, as it could be shown that in presence of phloridzin the R.Q. was lowered at the same period of incubation in which both carbohydrate disappearance and respiration were inhibited. Here again the lowering of the R.Q. by phloridzin falls in line with similar observations made *in vivo* [Bonsignore & Cavaglione, 1936]. These workers, using guinea-pigs, observed no increase, when glucose was administered, of the R.Q. lowered by phloridzin poisoning.

The almost toxic behaviour of phloretin in liver slices of starved rats is particularly interesting as it was possible to reverse its inhibitory effect on carbohydrate synthesis by adding glucose, in fact to make it behave like phloridzin. It seems feasible to assume that phloretin administered to the body may

have carbohydrate at its disposal, and may therefore be able to produce physiological effects similar to those of phloridzin. This is in accordance with the findings of Lambrechts [1936] who observed glucosuria following the administration of phloretin to chloralosed dogs.

SUMMARY

1. Carbohydrate synthesis in liver slices is partly suppressed by NaF and iodoacetate but is apparently increased in presence of phloridzin. The increase was shown to be the result of an inhibition of carbohydrate disappearance by phloridzin in the early stage of incubation. Evidence was produced that this disappearance, which was unaffected by insulin, was due to oxidation rather than to fermentation of the carbohydrate. Hence it was concluded that phloridzin inhibits carbohydrate oxidation *in vitro*.

2. The above view was supported by a regularly occurring inhibition of O_2 consumption by phloridzin at the same period of incubation at which in its absence carbohydrate oxidation mainly takes place. Further evidence for this view was given by the fact that the R.Q. for the above period approached unity in absence and was found to be 0·58 in presence of phloridzin.

3. Phloretin, unlike phloridzin, partly inhibits both O_2 uptake and carbohydrate synthesis in liver slices in concentration higher than 0·001 *M*. But when glucose was added simultaneously, phloretin showed an effect similar to that of phloridzin on carbohydrate synthesis. This indicates that the phloridzin effect is probably due to the complete glucoside rather than to its constituents.

4. The above phenomenon and further the fact that the carbohydrate level of liver tissue remained unchanged when incubated with phloridzin under anaerobic conditions dispose of the idea of a possible hydrolysis of the glucoside during the period of incubation, which otherwise could have vitiated the observations.

I wish to express my thanks to Sir F. G. Hopkins for his interest in this work.

REFERENCES

Allen (1913). Studies concerning glycosuria and diabetes. Cambridge.
Ashford & Holmes (1929). *Biochem. J.* **23**, 748.
Bach & Holmes (1937). *Biochem. J.* **31**, 89.
Bonsignore & Cavaglione (1936). *Bioch. terap. sper.* **23** 106.
Dann & Quastel (1928). *Biochem. J.* **22**, 245.
Dische & Popper (1926). *Biochem. Z.* **175**, 371.
Dixon & Keilin (1933). *Biochem. J.* **27**, 86.
Erlandsen (1910). *Biochem. Z.* **23**, 329.
Fiske & Subbarrow (1925). *J. biol. Chem.* **66**, 375.
Fleischmann (1937). *Biochem. Z.* **291**, 415.
Friedemann, Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
Goldstein, Tatelbaum, Ehre & Murlin (1932). *Amer. J. Physiol.* **101**, 166.
Haarmann & Brink (1935). *Biochem. Z.* **282**, 434.
Hagedorn & Jensen (1923). *Biochem. Z.* **135**, 46.
Lambrechts (1936). *C.R. Soc. Biol., Paris*, **121**, 870.
Laser & Rothschild (1939). *Proc. roy. Soc.* B, **126**, 539.
Lundsgaard (1933). *Biochem. Z.* **264**, 209.
Nash & Benedict (1923). *J. biol. Chem.* **55**, 755.
Rathery, Kouzilsky & Gibert (1930). *C.R. Soc. Biol., Paris*, **103**, 472.
Rona, Mislowitzer & Seidenberg (1925). *Biochem. Z.* **162**, 87.
Schwarz & Sassler (1928). *Biochem. Z.* **198**, 250.
Tanko (1931). *Biochem. Z.* **239**, 318.
Toerell & Norberg (1932). *Biochem. Z.* **249**, 53.
Underhill (1912). *J. biol. Chem.* **13**, 15.
Verzár & Laszt (1934). *Biochem. Z.* **270**, 35.
Wilbrandt & Laszt (1933). *Biochem. Z.* **259**, 389.
Willstätter (1936). *Enzymologia*, **1**, 213.