The Inhibition of Photophosphorylation by Phlorizin and Closely Related Compounds*

G. Douglas Winget,† Seikichi Izawa,‡ and Norman E. Good

ABSTRACT: In isolated chloroplasts, adenosine triphosphate formation and the concomitant stimulation of electron transport are reversibly inhibited by phlorizin (4,4',6'-trihydroxy-2'-glucosidodihydrochalcone). Phlorizin does not inhibit that part of the electron transport which normally continues in the absence of phosphorylation nor does phlorizin inhibit the rapid nonphosphorylating electron transport induced by uncouplers such as methylamine, atebrin, and carbonyl cyanide 4-trifluoromethoxyphenylhydrazone. Chloroplasts uncoupled by treatment with ethylenediaminetetraacetic acid are also insensitive to phlorizin. Chloroplasts uncoupled by arsenate and adenosine diphosphate are sensitive to the inhibitor. Similar concentrations of phlorizin inhibit the light-triggered adenosine triphosphatase of chloroplasts. Phlorizin inhibition is weaker when the phosphorylation process is limited by low concentrations of adenosine diphosphate or low light intensity but much stronger when phosphorylation is limited by low phosphate concentrations. It therefore seems probably that phlorizin interferes with a reaction close to phosphate utilization. However, the interactions of phlorizin and phosphate are only semicompetitive. 4,6'-Dihydroxy-2'-glucosidodihydrochalcone (II), which has one less hydroxyl but is otherwise structurally identical with phlorizin, was prepared and found to be equally specific and about ten times more potent than phlorizin. A number of compounds with structural relationships to II were prepared and tested. These resorcyl analogs of II were much easier to synthesize than the corresponding phloroglucyl analogs of phlorizin and shared with II the increased potency. The position of the sugar on the "B" ring proved to be critical for specificity, since 2',4-dihydroxy-4'-glucosidodihydrochalcone (with the glucoside in a position *para* to the carbonyl rather than *ortho* as in II) inhibits electron transport whether the electron transport is associated with phosphorylation or not. In contrast considerable modification of the "A" ring is possible without affecting the specificity of inhibition. The hydroxyl group can be moved from the 4 position to the 2 position, methylated or removed entirely with no apparent change in the specificity and little change in activity relative to that shown by II. When the "A" ring is absent, however, as it is in the corresponding acetophenone glucosides, the compounds have much reduced activity. If the double bonds of the two ring systems of the molecule are conjugated, as in the chalcones, the molecule shows little if any inhibitory action. There seems to be no requirement for a specific sugar, since the galactoside corresponding to the glucoside, II, is equally active. However the presence of some sugar at the 2' position is required to confer specificity since the aglycones of phlorizin and its resorcyl analogs are equally inhibitory to phosphorylating and nonphosphorylating electron transport.

Electron transport dependent phosphorylation can be inhibited in three fundamentally different ways: by preventing electron transport, by uncoupling electron transport from phosphorylation, and by inhibiting the phosphorylation reaction itself. Inhibition of the last type has been called "energy transfer inhibition" meaning that the transfer of energy from the redox reactions of the electron transport chain to ATP is blocked (Sanadi, 1965). Several such inhibitors of energy transfer reactions in mitochondrial oxidative phosphorylation have been described, the best known being oligomycin (Lardy *et al.*, 1958). Lately three comparable inhibitors of photophosphorylation in chloroplasts have been described: Dio-9, an experimental antibiotic of unknown composition (McCarty *et al.*, 1965); dicyclohexylcarbodiimide (McCarty and Racker, 1967); and the well-known glucosuria-producing substance, phlorizin (Izawa *et al.*, 1966).

Phlorizin has long been known to animal physiologists as an inhibitor of the transport of glucose across cell membranes (see Crane, 1960), as an inhibitor of several transphosphorylation reactions (Meyerhof and Wilson, 1948; Doudoroff, 1943; Cori *et al.*, 1939), as an effective but little understood modifier of mitochondrial reactions (Keller and Lotspeich, 1959). Consequently its involvement in yet another phosphorylation reaction, the phosphorylation of ADP, is of particular interest. The studies described in this paper were undertaken in order to define more precisely the nature of the inhibition of photophosphorylation and to

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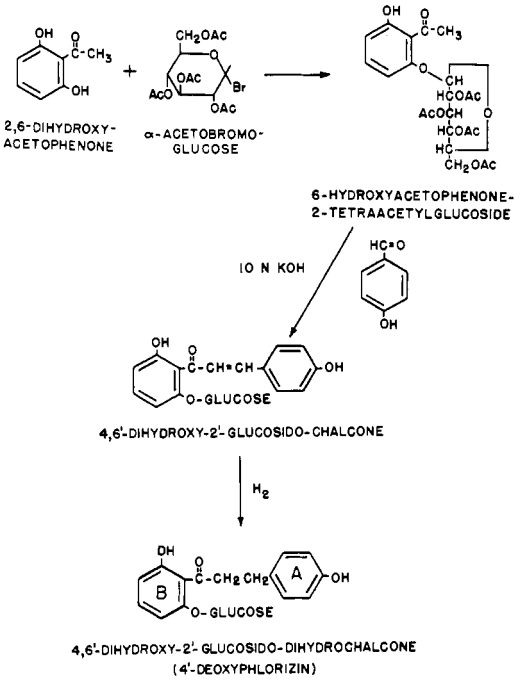


FIGURE 1: Synthesis of 4'-deoxyphlorizin.

determine, if possible, the relation of the structure of phlorizin to its activity in chloroplasts. We were particularly intrigued by the latter problem since phlorizin is a glucoside and so many of the reactions known to be affected by phlorizin involve either glucose or glucose and phosphate.

Syntheses of Phlorizin Analogs

A very large number of different dihydrochalcones, all in a sense analogs of phlorizin, may be prepared by varying the position, number, and kinds of substituents of the two rings. We have prepared several such analogs as described below. Their structures are shown on Figure 5 and 6 and Table II which describe their effects on electron transport and phosphorylation in chloroplasts.

There is some confusion in the literature as to the numbering of the substituent groups on the two aromatic rings of phlorizin and chalcones. In this report the position of groups attached to the ring labeled "A" (see Figure 1) will be designated by numbers while that labeled "B" will be designated by primed numbers. In all instances "B" refers to the phloroglucyl or resorcyl portion of the molecule.

α-Acetobromoglucose was prepared by the procedure of Barczai-Martos and Korsoy (1950).

α-Acetobromogalactose was prepared by treating purified galactose pentaacetate in acetic acid with dry hydrogen bromide according to the procedure of Hanson *et al.* (1955). Galactose pentaacetate was generously contributed by Dr. R. G. Hanson.

2'-6'-Dihydroxyacetophenone was obtained from Aldrich Chemical Co., Milwaukee, Wis. 2',4'-Dihydroxyacetophenone was from Distillation Products Industries, Rochester, N. Y. Salicylaldehyde, benzaldehyde, and *p*-anisaldehyde were from Matheson Colman and Bell.

Phloretin was prepared by mild acid hydrolysis of phlorizin. It was treated with charcoal (Norit A) and recrystallized from aqueous alcohol. Phlorizin itself (from Nutritional Biochemicals) was similarly treated with Norit A and recrystallized from hot water.

Melting points were determined on a Fisher-Johns melting point block which had been calibrated with pure standard compounds. Carbon–hydrogen analyses were performed by Galbraith Laboratories Inc., Knoxville, Tenn.

Preparation of 4,6'-Dihydroxy-2'-glucosidodihydrochalcone (4'-Deoxyphlorizin) (II). The sequence of reactions for preparation of 4'-deoxyphlorizin is shown schematically in Figure 1. Comparable reaction sequences were used in the preparation of the other phlorizin analogs.

A. 2'-(2,3,4,6-Tetraacetylglucosido)-6'-hydroxyacetophenone. 2',6'-Dihydroxyacetophenone (5.1 g, 34 mmoles) was dissolved in 38 ml of cold (0°), dry acetone. To this solution, 16.7 g of acetobromoglucose (41 mmoles) was added slowly with stirring. Then 19 ml of cold 2 M NaOH was added slowly with stirring. After 45 min the solution was removed from the ice bath, 37 ml of acetone was added, and the solution was allowed to stand 24 hr. The tetraacetyl glucoside readily crystallized from the reaction mixture and separated by filtration, giving 5.8 g (36% theory) crude product. When recrystallized from hot methanol the product had a melting range of 200–202°, lit. (Diedrich, 1962) mp 201–203°. This compound was also a starting material for the preparation of three other dihydrochalcones. The tetraacetyl glucoside (3.4 g, 7 mmoles) was treated with 10 N KOH for 20 min in the cold (0°). After this saponification, the alkaline solution was passed over a carboxylic acid ion-exchange resin in the acid form. The eluate was taken to a small volume from which 2.1 g (86% theory) of 6'-hydroxy-2'-glucosidoacetophenone crystallized. The white needles melted at 173–176°.

B. 4,6'-Dihydroxy-2'-glucosidochalcone. The tetraacetyl glucoside described above (3.4 g, 7 mmoles) was slurried with 3 ml of ethanol and treated with 18 ml of 10 N KOH for 10 min while in an ice bath. After this saponification, *p*-hydroxybenzaldehyde (1.05 g, 8 mmoles) was added. After 3 days at room temperature, the solution was acidified (to pH 5) with cold 3 N HCl. The chalcone glucoside readily crystallized and was collected by filtration. The crude product (2.9 g, mp 163–168°) was recrystallized from 50% ethanol giving 2.7 g (85% theory) fine yellow crystals, mp 170–171°, lit. (Diedrich, 1962) mp 170–171°.

C. 4,6'-Dihydroxy-2'-glucosidodihydrochalcone (4'-deoxyphlorizin) (II). An alcoholic solution of the chalcone (0.42 g, 1 mmole) was treated with hydrogen gas in the presence of 0.2 g of 10% palladium on charcoal at room temperature and atmospheric pressure. The compound was allowed to react with approximately the theoretical amount of hydrogen. After hydrogenation was complete, the catalyst was filtered and the solution was diluted with an equal volume of water. The product crystallized freely in the cold. After recrystallization

from aqueous ethanol there was 0.38 g (90% theory) of fine white crystals, mp 133–134°, lit. (Diedrich, 1962) mp 134–136°.

Preparation of 2',4-Dihydroxy-4'-glucosidodihydrochalcone (III). A. 4'-(2,3,4,6-TETRAACETYLGLUCOSIDO)-2'-HYDROXYACETOPHENONE. 2',4'-Dihydroxyacetophenone (5.1 g, 34 mmoles) dissolved in 38 ml cold, dry acetone was allowed to react with acetobromoglucose (16.5 g, 40 mmoles) and 2 M NaOH as described above. After 24 hr, the acetone was removed at reduced pressure, the organic phase was washed several times with 50-ml portions of water, then crystallized from hot 60% methanol. The yield was 2.4 g (15% theory), mp 113–120°, lit. (Reichel and Steudel, 1942) mp 130–131°

B. 2',4-DIHYDROXY-4'-GLUCOSIDOCHALCONE. The tetraacetyl glucoside above (0.96 g, 1 mmole) was stirred with 3 ml of 95% ethanol and 5.1 ml of 10 N KOH for 10 min in an ice–water bath. After this saponification, 0.3 g (2.3 mmoles) of *p*-hydroxybenzaldehyde was added. After 5 days at room temperature, the solution was neutralized (to pH 5.0) with cold 3 N HCl. A very fine yellow precipitate was collected by centrifugation, then dissolved in hot 50% ethanol from which it readily crystallized on cooling. The yield was 0.315 g (38% theory), mp 212–214°, lit. (Jorio, 1959) mp 222°.

C. 2',4-DIHYDROXY-4'-GLUCOSIDODIHYDROCHALCONE (III). The above chalcone (0.21 g, 0.5 mmole) dissolved in ethanol was hydrogenated in the presence of 0.2 g of 10% palladium-on-charcoal catalyst with hydrogen gas at atmospheric pressure. Approximately the theoretical amount of hydrogen was consumed in the reaction and after filtration of the charcoal, dilution of the alcoholic solution with water, the reaction gave 0.08 g (39% theory) of product melting at 154–155°, lit. (Jorio, 1959) mp 157°.

Preparation of 2,6'-Dihydroxy-2'-glucosidodihydrochalcone (IV). A. 2,6'-DIHYDROXY-2'-GLUCOSIDOCHALCONE. This compound was prepared in the same way as the 4,6'-dihydroxy isomer (II) except that salicylaldehyde (*o*-hydroxybenzaldehyde) was substituted for *p*-hydroxybenzaldehyde, giving 2.4 g of product (78% theory) when recrystallized, mp 142–145°.

B. 2,6'-DIHYDROXY-2'-GLUCOSIDODIHYDROCHALCONE (IV). The chalcone above was reduced with hydrogen gas as previously described. After hydrogenation, the catalyst was filtered, and the alcoholic solution was taken to dryness. The compound was recrystallized from water giving 0.29 g (70% theory), mp 95–98°. *Anal.* Calcd for $C_{21}H_{24}O_9$: C, 60.0; H, 5.8. Found: C, 57.8; H, 5.9.

Preparation of 6'-Hydroxy-2'-glucosidodihydrochalcone (VI). A. 6'-HYDROXY-2'-GLUCOSIDOCHALCONE. This compound was prepared from the tetraacetyl glucoside and benzaldehyde using the same procedure as used for the similar compounds, II and IV. The reaction mixture oiled out on acidification, and the oil was separated from the aqueous phase. The oil, when thoroughly dry, gave an amorphous solid melting the range of 203–210°. Attempts to recrystallize this substance failed, so the crude solid was used for subsequent preparation of the dihydrochalcone.

B. 6'-HYDROXY-2'-GLUCOSIDODIHYDROCHALCONE (VI). The crude product described above was hydrogenated employing the same procedure as previously used. It took up eight-tenth times the theoretical amount of hydrogen. Addition of water to the alcohol solution gave an oil which subsequently crystallized. The compound was recrystallized several times from aqueous ethanol, giving a fine white powder, mp 93–95°. Although the compound was obviously impure (see analysis) its biological activity was comparable with the activity of the above described relatively pure substances. When this observation and the method of synthesis are considered together, it seems highly probable that the material has the structure assigned. *Anal.* Calcd for $C_{21}H_{24}O_4$: C, 62.4; H, 6.0. Found: C, 59.8; H, 6.2.

Preparation of 4-Methoxy-6'-hydroxy-2'-glucosidodihydrochalcone (4-O-Methyl-4'-deoxyphlorizin) (V). A. 4-METHOXY-6'-HYDROXY-2'-GLUCOSIDOCHALCONE. 6'-Hydroxy-2'-glucosido-acetophenone (1.45 g, 4.1 mmoles) was treated with *p*-anisaldehyde (0.6 g, 4.5 mmoles) dissolved in 25 ml of 1 N NaOH. The flask was evacuated and tightly stoppered to reduce the oxygen concentration. After 3-days incubation with stirring the solution was acidified to pH 5. This was accompanied by an oiling out of a second phase. After several days the oil was heavy enough to be filtered. Attempts to recrystallize this oil failed, but it gave a solid when dry which appeared to melt around 126°.

B. 4-*O*-METHYL-4'-DEOXYPHLORIZIN (V). Catalytic hydrogenation of the crude chalcone (0.9 g) gave 0.6 g of the dihydrochalcone; pale yellow needles from aqueous ethanol, mp 106–108°. *Anal.* Calcd for $C_{22}H_{26}O_9$: C, 60.8; H, 6.0. Found: C, 60.6; H, 6.1.

Preparation of 4,6'-Dihydroxy-2'-galactosidodihydrochalcone (4'-Deoxyphloretin-2'-galactoside) (VII). A. 6'-HYDROXY-2'-TETRAACETYLGALACTOSIDOACETOPHENONE. This compound was prepared the same way as the glucose isomer, using acetobromogalactose in place of acetobromoglucose. The compound did not spontaneously crystallize as did the tetraacetyl glucoside, but was precipated from the acetone layer by stirring this layer into a large excess of ice–water. When recrystallized from methanol it melted at 122–124°.

The structure of this compound was confirmed in the following manner. Saponification in cold 10 N KOH followed by passage through a carboxylic acid ion-exchange resin in the acid form as described above, gave the free 6'-hydroxyacetophenone 2'-galactoside in 88% yield, mp 210–215°. Treatment of a portion of the galactoside with boiling 3 N HCl and separation of the solids after cooling gave the starting 2',6'-dihydroxyacetophenone (98% theory from the galactoside), mp 150–153°. The mixture melting point with starting material was not depressed.

B. 4'-DEOXYPHLORETIN-2'-GALACTOSIDE (VII). The reaction to prepare the chalcone was carried out as with the glucose isomer. The chalcone did not crystallize and therefore the preparation was evaporated to a small volume, dissolved in ethanol, and catalytically hydrogenated. The product (0.13 g, 10% theory) crystallized as long, pale yellow needles, having mp 106–108°. *Anal.*

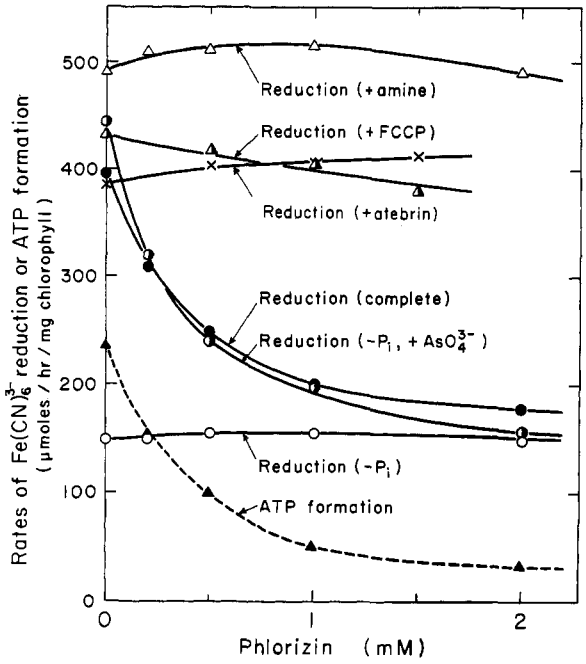


FIGURE 2: Effect of phlorizin on rates of ferricyanide reduction and ATP formation in illuminated chloroplasts. The reaction mixture (2.0 ml) contained in micromoles: sucrose, 200; Tricine buffer (pH 8.4), 100; potassium ferricyanide, 0.8; $MgCl_2$, 4; ADP, 2; [^{32}P]Na_2HPO_4, 30; or Na_2HAsO_4, 40; methylamine-HCl, 20; atebrin-HCl, 0.06; and carbonyl cyanide 4-trifluoromethoxyphenylhydrazone, 0.01 (if added). Chloroplasts in the reaction mixture contained 40 μg of chlorophyll. Saturating orange light (>560 mμ) was used.

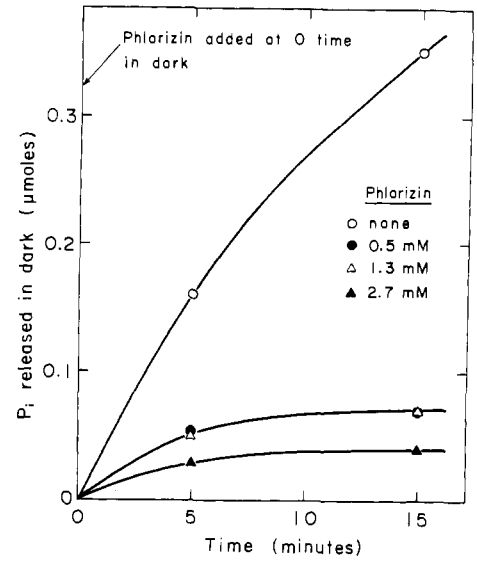


FIGURE 3: Effect of phlorizin on ATP hydrolysis by chloroplasts in the dark (light-triggered, Mg^{2+}- and sulfhydryl-requiring ATPase). The reaction mixture (9 ml) contained in μmoles: Tris-HCl (pH 8.0), 600; $MgCl_2$, 36; ADP, 22; PMS, 0.5; [^{32}P]Na_2HPO_4, 60; and chloroplasts (600 μg of chlorophyll). It was illuminated in a test tube with strong orange light (>560 mμ) until all the ADP was converted into labeled ATP (7 min). Then 3 ml of a cysteine solution (600 μmoles of cysteine) was added to the reaction mixture, and illumination was continued for a further 3 min to activate ATPase. Immediately after this an aliquot (1 ml) was taken for analysis of the ATP level at zero time, then the light was turned off and additional 2-ml aliquots were quickly transferred to test tubes containing various amounts of phlorizin dissolved in 1 ml of Tris-HCl (pH 8.0, 50 mM). Aliquots (1 ml) were then taken from each test tube, at the time intervals indicated, and analyzed for labeled ATP.

Calcd for $C_{21}H_{24}O_9$: C, 60.1; H, 5.8. Found: C, 60.2; H, 6.0.

Effects of Phlorizin and Its Analogs on Electron Transport and Phosphorylation in Chloroplasts

Assay Methods

Chloroplasts were isolated in the cold (0–3°) from leaves of spinach (*Spinacia oleracea* L) obtained from a local market. The leaves were washed with cold, distilled water and then ground briefly (5–10 sec) in a Waring Blendor in a medium containing 0.3 M NaCl, 1.0 mM $MgCl_2$, 1.0 mM disodium ethylenediaminetetraacetate, and 0.04 M TES[1] adjusted to pH 7.3 with NaOH. The brei was filtered through several layers of cheesecloth before centrifugation at 2000*g* for 5 min. The pellet was resuspended in a medium containing 0.2 M sucrose, 0.5 mM $MgCl_2$, and 0.03 M tricine adjusted to pH 7.3 with NaOH. Larger particles were removed by a brief centrifugation at 2000*g* for about 1 min, then the chloroplasts were sedimented by further centrifugation at 2000*g* for 5 min. The pellet was again suspended, sedimented, and finally resuspended in a small volume of the same medium. Chlorophyll concentration of this stock suspension was determined by the method of Arnon (1949).

Rates of ferricyanide reduction were recorded as changes in absorbance at 420 mμ using a modified Bausch & Lomb Spectronic 505 spectrophotometer (Izawa and Good, 1965).

ATP was measured as residual radioactivity in the aqueous phase after extraction of remaining ^{32}P-labeled orthophosphate as phosphomolybdate following the procedure of Avron (1960). The radioactivity was measured with an immersion Geiger tube (type M-6, 20th Century Electronics, Surrey, England). A non-illuminated reaction mixture was used as blank. However corrections for the blank were rarely necessary since it usually represented less than 1% of the number of counts expected in the ATP, or less than 0.01% of the counts in the added orthophosphate.

Results

A. Phlorizin. Figure 2 illustrates the effects of various concentrations of phlorizin on electron transport and phosphorylation in chloroplasts. The part of the electron transport which is independent of phosphorylation, that is, the electron transport which occurs in the absence of phosphate, is totally insensitive to phlorizin. Electron transport uncoupled from phosphorylation by methylamine, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone, or atebrin is almost equally insensitive. However that part of the electron

[1] Abbreviations used are: TES, tris(hydroxymethyl)methylaminoethanesulfonic acid; tricine, tris(hydroxymethyl)methylglycine.

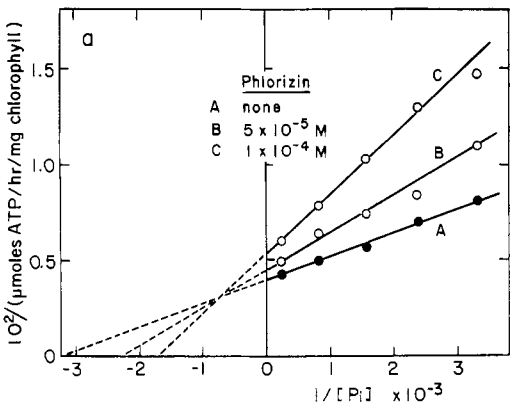


FIGURE 4: Lineweaver–Burk plots of the kinetics of phlorizin inhibition of ATP formation with limiting phosphate. Experimental conditions as in the figure except for variations in the concentration of phosphate. Note that the per cent inhibition increases as the rate of phosphorylation decreases.

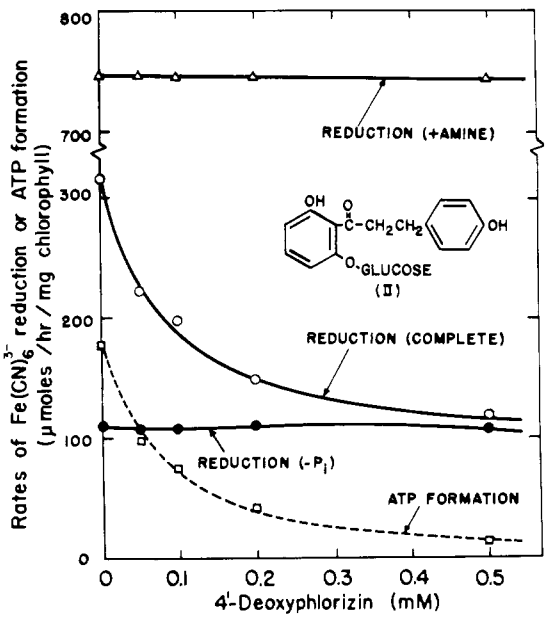


FIGURE 5: Effects of 4′-deoxyphlorizin on rates of electron transport and ATP formation in illuminated chloroplasts. Reaction conditions as in Figure 2.

transport which is dependent upon ADP and phosphate (or ADP and arsenate) is almost completely inhibited by 2×10^{-3} M phlorizin. Moreover the inhibition of ATP synthesis exactly parallels the inhibition of the phosphorylation-dependent electron transport.

Table I shows that chloroplasts uncoupled by EDTA treatment are only slightly sensitive to phlorizin. Since EDTA uncouples by removing a coupling factor (Avron, 1963) which is a latent ATPase (Vambutas and Racker, 1965), the observation suggests that the reactions blocked by phlorizin are very close to the actual phosphorylation of ATP. This suggestion is also supported by the observation that phlorizin inhibits the ATPase activity of the coupling factor (R. E. McCarty, personal communication) and the ATPase activity of appropriately activated chloroplasts (Figure 3).

Chloroplasts seem less sensitive to phlorizin when electron transport and phosphorylation are limited by low light intensities or low ADP concentrations. This is probably no more than an expression of the decreased importance of the phlorizin-sensitive step as a rate-determining factor when other limiting steps are introduced. However the opposite is true when phosphorylation is limited by suboptimal concentrations of phosphate. Under these conditions sensitivity to phlorizin is greatly increased but, as Figure 4 shows, the inhibition by phlorizin is only partially competitive with phosphate. It is not clear whether phlorizin inhibits the utilization of phosphate noncompetitively or inhibits an enzyme which uses a bound form of phosphate. Nevertheless it does seem probable that phlorizin inhibits some reaction close to the uptake or utilization of phosphate.

Phlorizin-induced inhibition of phosphorylation is almost completely reversed when the chloroplasts are washed free of inhibitor.

"Noncyclic" phosphorylation and "cyclic" phosphorylation with phenazine methosulfate (PMS) as electron carrier are about equally sensitive to phlorizin.

B. Phlorizin Analogs. 4′-Deoxyphlorizin (II), which differs from phlorizin in lacking a hydroxyl group *para* to the carbonyl group, inhibits photophosphorylation and phosphorylation-dependent electron transport in much the same manner as phlorizin (see Figure 5). Again nonphosphorylating electron transport, whether "basal" or amine uncoupled, is totally insensitive. And again the inhibition of ATP synthesis closely parallels the inhibition of the extra electron transport which is associated with phosphorylation. 4′-Deoxyphlorizin is about ten times more active than phlorizin in inhibiting phosphorylation.

2,6′-Dihydroxy-2-glucosidodihydrochalcone (IV), which differs from II only in the position of the hydroxyl in the "A" ring, is equally specific and equally potent (see Table II).

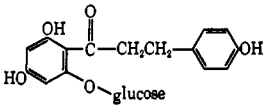
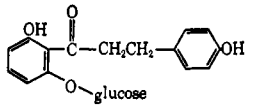
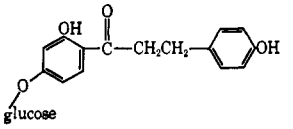
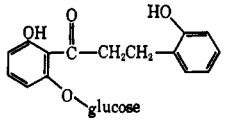
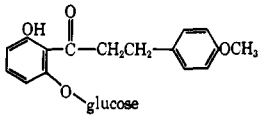
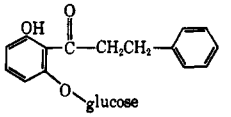
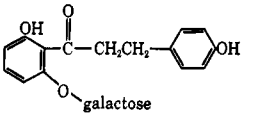
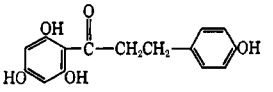
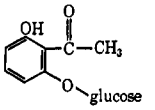
4-*O*-Methyl-4′-deoxyphlorizin (V) which differs from 4′-deoxyphlorizin only in having the 4-hydroxy group methylated is again equally specific and equally potent (see Table II).

TABLE I: Effect of Phlorizin on EDTA-Uncoupled Chloroplasts.[a]

Phlorizin (mM)	Ferricyanide Reduction (μmoles/hr per mg of chlorophyll)	Inhibition (% lowering of initial rate)
0	864	
0.5	750	13
1.0	696	19
1.5	702	19

[a] Chloroplasts were pretreated with 2 mM EDTA in a low salt medium (5 mM tricine, pH 7.3) for 1 hr at 0°, and the reaction was run at pH 7.4.

TABLE II: Inhibition by Phlorizin and Analogs.[a]

Code No.	Compound[b]	Concentration (M) Giving 50% Inhibition			
		Electron Transport			
		Nonphosphorylating (Basal)	Amine Uncoupled	Phosphorylation Coupled[c]	ATP Formation
I		No effect	No effect	3×10^{-4}	3×10^{-4}
II		No effect	No effect	6×10^{-5}	6×10^{-5}
III		1.5×10^{-3}	1×10^{-3}	5×10^{-4}	
IV		No effect	No effect	4×10^{-5}	5×10^{-5}
V		No effect	No effect	3×10^{-5}	3×10^{-5}
VI		No effect	No effect	1.5×10^{-4}	*d*
VII		No effect	No effect	1×10^{-5}	1×10^{-5}
VIII		$>5 \times 10^{-4}$	4×10^{-4}	4×10^{-4}	
IX		No effect		$>1 \times 10^{-3}$	

[a] See Figure 2 for reaction conditions. [b] I, phlorizin; II, 4′-deoxyphlorizin; III, 2′,4-dihydroxy-4′-glucosidodihydrochalcone; IV, 2,6′-dihydroxy-2′-glucosidodihydrochalcone; V, 4-*O*-methyl-4′-deoxyphlorizin; VI, 6′-hydroxy-2′-glucosidodihydrochalcone; VII, 4′-deoxyphloretin-2′-galactoside; VIII, phloretin; IX, 6′-hydroxy-2-glucosidoacetophenone. [c] That part of the electron transport which depends upon the addition of ADP and phosphate. [d] Substance impure.

6′-Hydroxy-2′-glucosido-dihydrochalcone (VI), which differs from 4′-deoxyphlorizin in having no substitutions on the "A" ring, also seems to be about equally specific and equally potent (see Table II). This substance was impure (see analysis in section dealing with synthesis) and consequently the slightly lower activity and the small inhibition of the amine-uncoupled electron transport may not be characteristic of the pure substance.

4′-Deoxyphloretin-2-galactoside (VII), which differed from 4′-deoxyphlorizin only in having the glucose moiety replaced by galactose, inhibited electron transport and phosphorylation in the same way. As Table II shows, it was neither more nor less specific than the glucose isomer and was approximately equal in activity.

Phlorizin and all of the above-mentioned analogs, II–VII, have in common (a) a free hydroxyl *ortho* to the carbonyl group and (b) a sugar moiety also *ortho* to

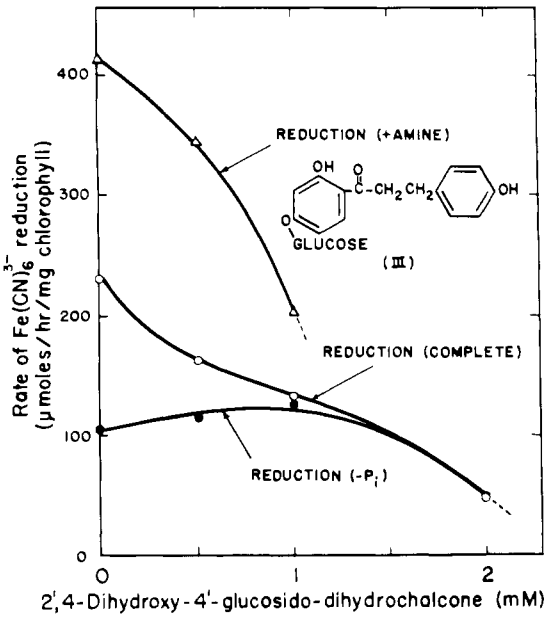


FIGURE 6: Effects of 2',4-dihydroxy-4'-glucosidodihydrochalcone on phosphorylating and nonphosphorylating electron transport. Note that both processes are sensitive to the inhibitor. Reaction conditions as in Figure 2.

the carbonyl group. Both of these substituents on the "B" ring seem to be required for specificity of action. Thus, 2',4-dihydroxy-4'-glucosidodihydrochalcone (III), which has the glucose *para* instead of *ortho*, is an unspecific inhibitor of all types of electron transport, whether phosphorylating, nonphosphorylating (basal) or amine uncoupled (Figure 6). The same is true of phloretin (VIII), the aglucone of phlorizin (Table II), and the other aglucones.

2-Glucosido-6-hydroxyacetophenone (IX), which differs from 4'-deoxyphlorizin (II) in lacking the "A" ring and an adjacent methylene group, has only about 1% of the activity of II (Table II).

Discussion

Apparently phlorizin inhibits photophosphorylation at the level of the phosphorylation of ADP. In inhibiting phosphorylation it indirectly inhibits that part of the electron transport associated with phosphorylation, but it has practically no effect on nonphosphorylating electron transport. Phlorizin is not at all competitive with ADP but it seems partially competitive with phosphate; there is little evidence of competition at high phosphate concentrations but strong evidence of competition at low phosphate concentrations. The kinetics of the phlorizin–phosphate interaction could result from several mechanisms but, in any event, it seems likely that the site of phlorizin action is close to the site of phosphate involvement. Perhaps phlorizin inhibits noncompetitively an enzyme involved in phosphate utilization (if this enzyme is not normally rate determining). If so the apparent competition may be simply the result of the increasing importance of this enzyme as a rate-controlling factor when the phosphate concentration is lowered. Alternatively, phlorizin may inhibit, either competitively or noncompetitively, an enzyme which utilizes a bound form of phosphate.

A number of analogs of phlorizin inhibit photophosphorylation in a very similar manner. Indeed both the potency and specificity of the inhibition seem remarkably independent of most of the structural features of phlorizin. Within rather wide limits, the groups on the two ring structures may be modified or omitted without loss of activity or specificity. Omission of the 4'-hydroxyl group of phlorizin (the hydroxyl *para* to the carbonyl) actually increases activity by more than an order of magnitude. It is not clear whether this increase in activity represents an increased enzyme–inhibitor affinity or reflects a stronger partitioning of a less polar molecule into the lipoidal chloroplast. The same question can be asked even in the case of the very inactive acetophenone analog of deoxyphlorizin which lacks entirely the "A" ring and is thus preponderately polar. In other words we cannot even be sure that the "A" ring is necessary in terms of an enzyme–inhibitor interaction. Certainly it does not seem to make much difference what groups are on the "A" ring or where. The 4-hydroxy, 2-hydroxy, 4-methoxy, and unsubstituted "A" rings confer essentially the same activity.

In contrast the 2' position of the sugar moiety seems critical. Either removal of the sugar (as in phloretin and the corresponding resorcylaglucones) or its transfer to the 4' position as in 2',4-dihydroxy-4'-glucosidodihydrochalcone (III) converts specific inhibitors of phosphorylation and phosphorylating electron transport into unspecific inhibitors of all kinds of electron transport. Unfortunately we cannot decide from experiments of the kind reported here whether or not this loss of specificity is accompanied by a decrease of activity against the phosphorylating reaction itself. Electron transport is inhibited by these unspecific inhibitors and hence indirectly phosphorylation is inhibited; the extent to which phosphorylation may still be directly inhibited is therefore unknown.

Although a sugar is required at the 2' position for specific inhibition of phosphorylation, it does not seem to matter whether this sugar is glucose or galactose. Perhaps a sugar or similar polyhydric moiety is required in the 2' position because of a critical interaction (hydrogen bonding?) between one of the sugar hydroxyls and the carbonyl oxygen.

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Phosphorus Nuclear Magnetic Resonance Studies of Phosphoproteins and Phosphorylated Molecules. II. Chemical Nature of Phosphorus Atoms in α_s-Casein B and Phosvitin*

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance spectroscopy has been used to investigate the chemical nature of phosphorus atoms in hen's egg yolk phosvitin and bovine α_s-casein B. For concentrations as low as 0.05 M in phosphorus, phosphorus–proton coupling patterns of 40.5-MHz ^{31}P nmr spectra can be resolved with the use of a computer of average transients. Phosphorus–proton coupling constants for these two proteins, as well as for several other phosphorylated molecules of biological interest, fall in the range 5–8 Hz. The variation of ^{31}P chemical shifts with pH in the range 3–11, for model compounds such as *O*-phosphoserine, *O*-phosphothreonine, glucose 1-phosphate, diethyl phosphate, β-diphosphopyridine nucleotide, and sodium pyrophosphate, can be put into two categories: (i) the ^{31}P chemical shifts in compounds with phosphodiester and with symmetrically substituted pyrophosphate linkages remain essentially constant; (ii) for phosphomonoester compounds there are relatively large changes in ^{31}P chemical shifts (about 4 ppm) with change of pH from 3 to 9. From the nuclear spin–spin coupling pattern, the variation of the ^{31}P chemical shift with pH, and the values of ^{31}P chemical shift, and phosphorus–proton coupling constants, we conclude that most of the phosphate groups in both phosvitin and α_s-casein B are attached to seryl residues as monoesters. Reversible line-broadening phenomena, which are not yet fully explained, are observed for *O*-phosphothreonine, glucose 1-phosphate, phosvitin, and α_s-casein in certain pH ranges. In general it appears that the high-resolution ^{31}P nuclear magnetic resonance can yield explicit information about the nature and, in some cases, steric disposition, of groups neighboring phosphates in a protein, by way of the phosphorus–proton coupling patterns, ^{31}P line widths, and variations of ^{31}P chemical shift with pH.

In our previous communication, Ho and Kurland (1966) discussed the controversy surrounding the nature of the phosphate groups in bovine α_s-casein B, that is, whether these are in the form of monoesters, diesters, symmetrically substituted pyrophosphate, or some combination of the above. Chemical and enzymatic degradations have been used to study this problem in the past, but these methods suffer from inherent ambiguities, *viz.*, chemical degradation, usually involving partial acid hydrolysis in an attempt to isolate *O*-phosphoserine or other phosphorylated amino acids or peptides (Perlmann, 1955; Hofman, 1958), can give rise to O–N acyl shifts, particularly adjacent to seryl residues (Desuelle and Casal, 1948), and thus rearrange

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