Phloretin: An Inhibitor of Phosphate Transfer and Electron Flow in Spinach Chloroplasts*

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ABSTRACT: Phloretin, the aglucone of phlorizin, is shown to act with a dual effect on energy conversion of chloroplasts and subchloroplast fragments. This compound can inhibit energy transfer at low concentrations (0–50 μM) by inhibiting phosphorylation and electron flow simultaneously. At higher concentrations phosphorylation is more strongly inhibited than electron flow and P:2e values approach zero. Phloretin is shown to inhibit chloroplast transphosphorylation reactions including acid-induced adenosine triphosphate (ATP) synthesis and in inhibition of hydrolysis of ATP by the Ca^{2+}- and Mg^{2+}-ATPase enzymes of the chloroplast. The inhibition of electron flow by phloretin is shown to be pH dependent as it inhibits both coupled and basal electron flow in the ferricyanide Hill reaction at pH 8.2 (near the pH optimum of the phosphorylation reaction). The compound has no effect on the basal electron flow at those pH regimes where the phosphorylating enzymes are inactive and the nonphosphorylating energy conversion into the proton pump is favored. Phloretin was found to have no effect on the proton pump under conditions where basal electron flow was significantly inhibited.

The results show that phloretin inhibits photophosphorylation by interacting with both the transphosphorylating and electron transport systems at pH regimes where ATP synthesis can occur and that the glucose moiety of the phlorizin analogs is not necessary for inhibition of the transphosphorylating enzymes. The failure of phloretin to inhibit the proton pump at pH regimes where the basal electron flow is significantly inhibited suggests that nonphosphorylating energy conversion may be coupled to electron flow in a fundamentally different manner from that functional in phosphorylating energy conversion.

Osmotically intact thylakoid membranes have been shown to be essential for energy coupling in phosphorylation and the proton pump in spinach chloroplasts (Jagendorf and Uribe, 1966b; Heber, 1967; Uribe and Jagendorf, 1968) and a membrane-bound protein (coupling factor) has been shown to be an additional component required in these reactions (McCarty and Racker, 1966). Immunochemical (McCarty and Racker, 1966) and inhibitor (McCarty *et al.*, 1965; Izawa and Hind, 1967; McCarty and Racker, 1967, 1968) techniques have indicated that the function of the coupling factor, CF_1, as a transphosphorylating enzyme is distinct from its role in the catalysis of the light-induced proton uptake reaction.

Phlorizin (Izawa *et al.*, 1966), Dio-9[1] (McCarty *et al.*, 1965), and Synthalin (Gross *et al.*, 1968), which have been reported as energy-transfer inhibitors in chloroplasts, function by selectively inhibiting that electron flow associated with phosphorylation. They have no effect on the basal (nonphosphorylating) electron flow or the enhanced electron flow induced by uncouplers (McCarty *et al.*, 1965; Izawa *et al.*, 1966). These compounds function at the level of phosphorylation and have no effect on energy transfer into the proton pump (McCarty and Racker, 1966; Izawa and Hind, 1967). McCarty and Racker (1967) have described the action of a compound which uniquely alters the expression of energy conversion in chloroplasts. Dicyclohexylcarbodiimide acts as an energy-transfer inhibitor when present at 0–25 μM and as an uncoupler at higher concentrations; it also inhibits basal and uncoupled electron flow. This compound does not inhibit the light-induced pH rise reaction and indeed it can reconstitute the reaction in chloroplast fragments which have lost this function due to partial removal of their coupling factor by treatment with EDTA.

It is probable that the above energy-transfer inhibitors exert their effect by interaction with membrane-localized components essential to energy flow. The described reconstitution of the pH rise reaction in EDTA-treated chloroplasts by dicyclohexylcarbodiimide suggested that such a chemical might be useful in studying site-specific interactions of chloroplast membranes with agents which can modify energy conversion; however, the lipophilic nature of dicyclohexylcarbodiimide renders it rather unsuitable for such studies and thus it seemed desirable to test other compounds of similar biological activity with the aim of achieving a more specific interaction with the energy conserving system of the chloroplast.

Phloretin, the aglucone of phlorizin, was described by Izawa *et al.* (1966) as an inhibitor of photophosphorylation and coupled and basal electron flow in spinach chloroplasts. The inhibition reported in their work was qualitatively similar to that reported for dicyclohexylcarbodiimide and it was therefore of interest to examine the effect of phloretin on various energy-linked reactions of chloroplasts in order to determine if it might be a useful tool in the study of membrane function in energy coupling.

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[1] Abbreviations used are: Synthalin, decamethylene diguanide; Dio-9, an experimental antibiotic; Tricine, tris(hydroxymethyl)methylglycine; Cl_2PhMe_2U, dichlorophenyl-1,1-dimethylurea; PMB, *p*-mercuribenzoate.

Chloroplast Preparations. Spinach chloroplasts were prepared from market spinach as described by Hind and Jagendorf (1963) and resuspended in 0.35 M NaCl containing 0.01 M Tricine (pH 8.0), when used for measurement of photophosphorylation and ATPase activity. When chloroplasts were to be used in acid-induced ATP synthesis, light-induced pH rise reactions, or cyclic photophosphorylation the chloroplasts were prepared as described by Jagendorf and Uribe (1966a,b). Chlorophyll was determined by the method of Arnon (1949).

Assays. Ferricyanide reduction in the presence of phloretin was determined by measuring the light-induced change in optical density at 420 nm by the method described by Avron *et al.* (1958). The accompanying phosphorylation was determined by the method of Avron (1960). Reaction mixtures of 2.0-ml volume contained in μmoles; Tricine, 80, at the pH indicated; $K_3Fe(CN)_6$, 1; $NaKHPO_4$, 10; ADP, 6; $MgCl_2$, 10; [^{32}P]P_i containing approximately 5×10^5 cpm; and phloretin at the concentrations indicated. The reactions included chloroplasts containing 34–100 μg of chlorophyll. Chlorophyll concentration curves showed that the reactions containing the highest chloroplast concentrations were saturated by white light from two tungsten lamps which provided energy of 6×10^5 ergs/cm^2 per sec at the surface of the reaction vessels. Dark controls were included for each phloretin concentration tested. Reactions were stopped by the addition of trichloroacetic acid to a final concentration of 2.5%.

Acid-induced ATP synthesis reactions were run as described by Jagendorf and Uribe (1966a,b) with the 0.9 ml of acid stage containing succinic acid (10 μmoles), Cl_2PhMe_2U (30 mμmoles), and chloroplasts containing 250 μg of chlorophyll. The basic stage contained Tricine (100 μmoles, pH 8.4), $NaKHPO_4$ (2 μmoles), ADP (0.2 μmole), $MgCl_2$ (5 μmoles), and NaOH to neutralize the succinic acid of the acid stage in a volume of 0.9 ml. Phloretin was included in either the acid or the basic stages, with the concentrations shown indicating in all cases the final concentration achieved after pH transition.

The effect of phloretin on the light activation and ATPase activity of the light-triggered Mg^{2+}-dependent ATPase of the chloroplast was determined using 1-ml reaction mixtures which contained in μmoles; Tris, 60, pH 8.0; $MgCl_2$, 5; ATP, 5; NaCl, 40; pyocyanine, 0.05; and dithiothreitol, 2. Chloroplasts containing 10 μg of chlorophyll and phloretin at the concentrations indicated were present in the reaction mixtures. The effect of phloretin on the hydrolysis of ATP by the light activated ATPase was determined in a two-step reaction. Chloroplasts were activated in a stirred 5-ml reaction mixture containing in μmoles; Tris, 300; pH 8.0; pyocyanine, 0.25; NaCl, 200; dithiothreitol, 10; and chloroplasts containing 500 μg of chlorophyll. After activation, 0.1-ml aliquots were removed to tubes containing in μmoles; Tris, 60, pH 8.0; $MgCl_2$, 5; ATP, 5; NaCl; 40; and phloretin at the concentrations indicated in a volume of 0.9 ml. Activations for both experiments were carried out at room temperature by illuminating with white light of intensity 3.2×10^5 ergs/cm per sec. ATP hydrolysis was allowed to proceed for 30 min in the dark at 35° and the reactions were terminated by the addition of trichloroacetic acid to a final concentration of 2%. The phosphate released was assayed by the method of Taussky and Shorr (1953).

The soluble Ca^{2+}-dependent ATPase of spinach chloroplasts was prepared by the method of McCarty and Racker (1968) using an activation mixture which contained in μmoles; Tris, 200, pH 8.0; dithiothreitol, 250; ATP, 5; and chloroplasts containing 1.0 mg of chlorophyll in a volume of 5 ml. The chloroplasts were incubated at room temperature for 2 hr, recovered by centrifugation at 1400*g* for 10 min, and then resuspended in 1 mM EDTA (pH 8.0) containing 0.1 mM ATP and allowed to stand for 15 min at room temperature. The activated soluble Ca^{2+}-ATPase was recovered as the supernatant of a centrifugation for 15 min at 41,000*g*. Assays for ATPase activity were carried out in reaction mixtures of 1.0-ml volume containing in μmoles; Tris, 60, pH 8.0; $CaCl_2$, 5; ATP, 5; NaCl, 40; and supernatant containing enzyme from the equivalent of 40 μg of chlorophyll.

Phloretin was prepared by the mild acid hydrolysis of phlorizin by the method of Muller and Robertson (1933) and recrystallized from ethanol–water, mp 268–270°, lit. mp (Fischer and Nouri, 1917) 264–271°.

Results

Effect of Phloretin on Ferricyanide Reduction and Coupled Phosphorylation. The addition of phloretin to reactions catalyzing coupled reduction of ferricyanide at pH 8.2 results in simultaneous inhibition of electron flow and phosphorylation. Thus at concentrations up to 0.05 mM the P:2e ratio remains relatively constant (Table I). At higher concentrations phosphorylation is more severely inhibited and the P:2e ratio ultimately approaches zero at the highest concentration tested. The inhibition of coupled electron flow and phosphorylation at pH 8.2 by phloretin is, therefore, essentially identical with that reported by McCarty and Racker (1967) for dicyclohexylcarbodiimide. At pH 8.2 phloretin action is twofold (Figure 1), with electron flow being inhibited in both the basal (minus P_i) and coupled (plus P_i) reactions. A similar inhibition pattern for PMB has been reported by Izawa and Good (1968). Phloretin,

TABLE I: Effect of Phloretin on Electron Transport and Coupled Phosphorylation.[a]

Phloretin (μM)	Ferricyanide Reduced (μequiv)	ATP Formed (μmoles)	P:2e
0	0.239	0.199	0.83
25	0.188	0.149	0.79
50	0.169	0.133	0.79
100	0.153	0.113	0.74
200	0.133	0.073	0.55
400	0.086	0.025	0.29
650	0.068	0.010	0.15

[a] Reactions were run as described in experimental procedures, whole chloroplasts containing 34 μg of chlorophyll were used in each reaction.

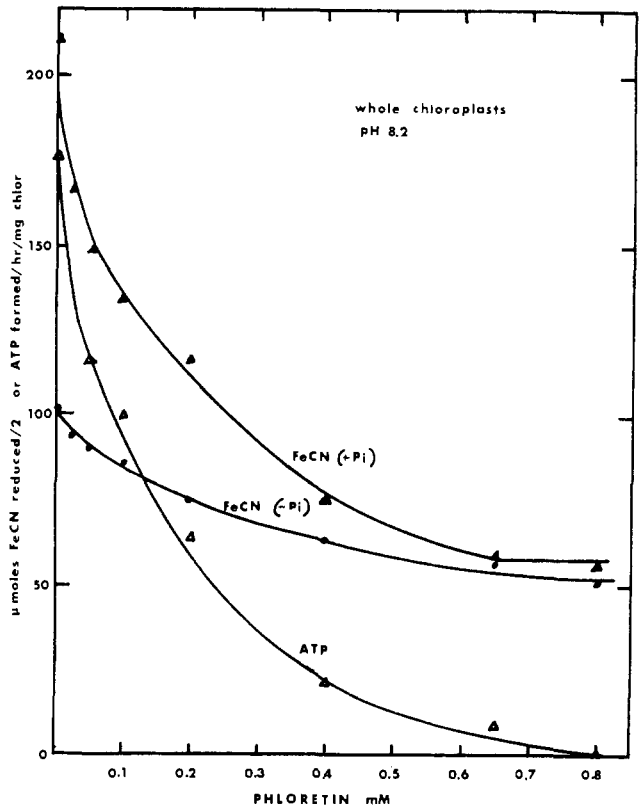

FIGURE 1: Effect of phloretin on coupled and basal electron flow at pH 8.2. Whole chloroplasts containing 34 μg of chlorophyll were used in reactions run as described in experimental procedures.

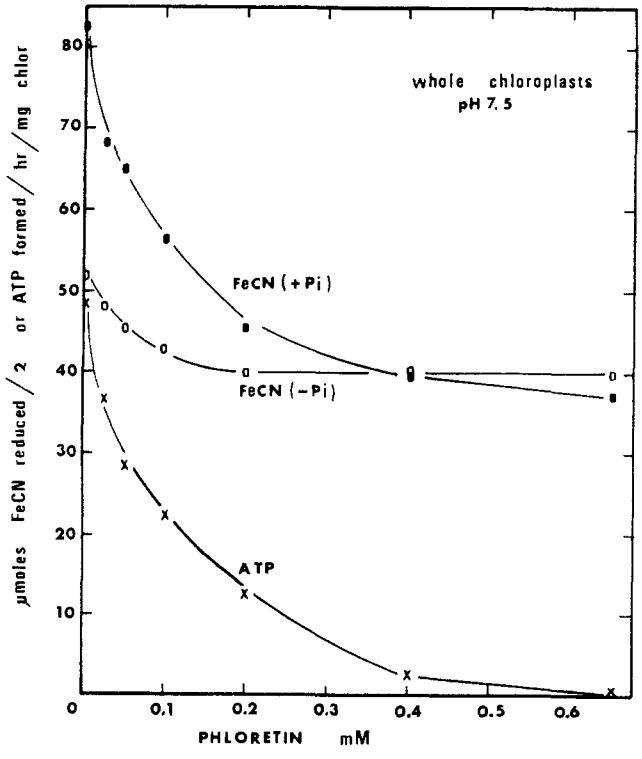

FIGURE 2: Effect of phloretin on coupled and basal electron flow at pH 7.5. Whole chloroplasts containing 100 μg of chlorophyll were used in reactions run as described in experimental procedures.

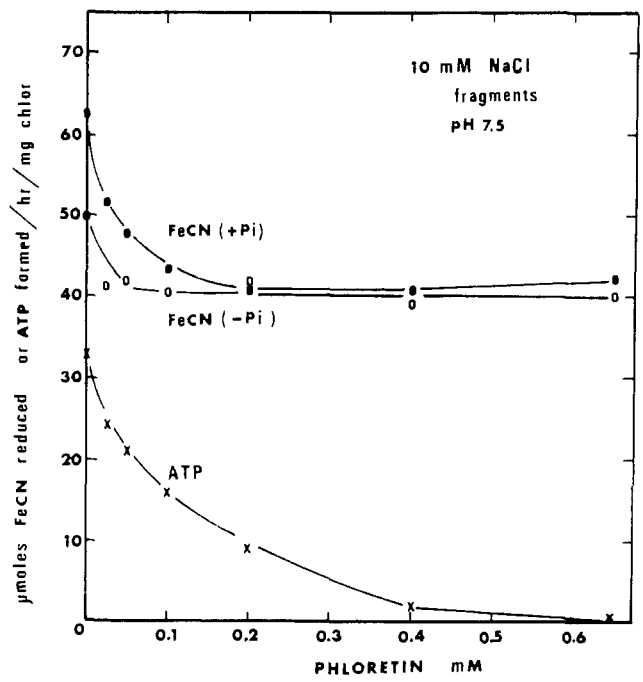

FIGURE 3: Effect of phloretin on coupled and basal electron flow at pH 7.5. Reaction conditions as for Figure 2 substituting chloroplast fragments for whole chloroplasts.

like dicyclohexylcarbodiimide, inhibits methylamine-uncoupled electron flow (E. G. Uribe, unpublished). These effects are clearly distinct from the usual action of energy-transfer inhibitors which only inhibit coupled electron flow; however, when the experiment is performed at pH 7.5 (Figure 2) one observes a different pattern of inhibition. The basal rate of electron flow shows a slight decrease and then no further inhibition as concentration is increased. This initial inhibition is not due to a small residual phosphorylating activity due to traces of endogenous phosphate as the same pattern of inhibition is observed when washed chloroplast fragments prepared by hypotonic shock (Jagendorf and Uribe, 1966a,b) are used in the reactions (Figure 3). The experiments of Figures 2 and 3 show that the two types of chloroplast preparations exhibit different sensitivities to the inhibitor. The basal electron transport of chloroplast fragments is inhibited about 20% by 0.2 mM or higher concentrations of phloretin while the final 20% level of inhibition requires at least 0.4 mM phloretin in whole chloroplasts. This increased sensitivity of chloroplasts fragments to low concentrations of phloretin was observed at pH 8.2, 7.5, and 7.0. Figures 2 and 3 reveal that when phloretin acts as an inhibitor of electron flow at pH 7.5 its action more closely approximates the inhibition by phlorizin, Dio-9, and Synthalin, which inhibit coupled electron flow to the level of the basal rate.

Figure 4 further illustrates the pH dependence of the phloretin inhibition of electron flow. Note that at pH 7.0 the rate of the coupled reaction is identical with the basal rate and there is no enhancement of electron flow by P_i, while the very slight residual ATP synthesis is inhibited as at higher pH regimes. Under these conditions phloretin has no inhibitory effect on electron transport in either system and thus at a pH at which there is almost no phosphorylating

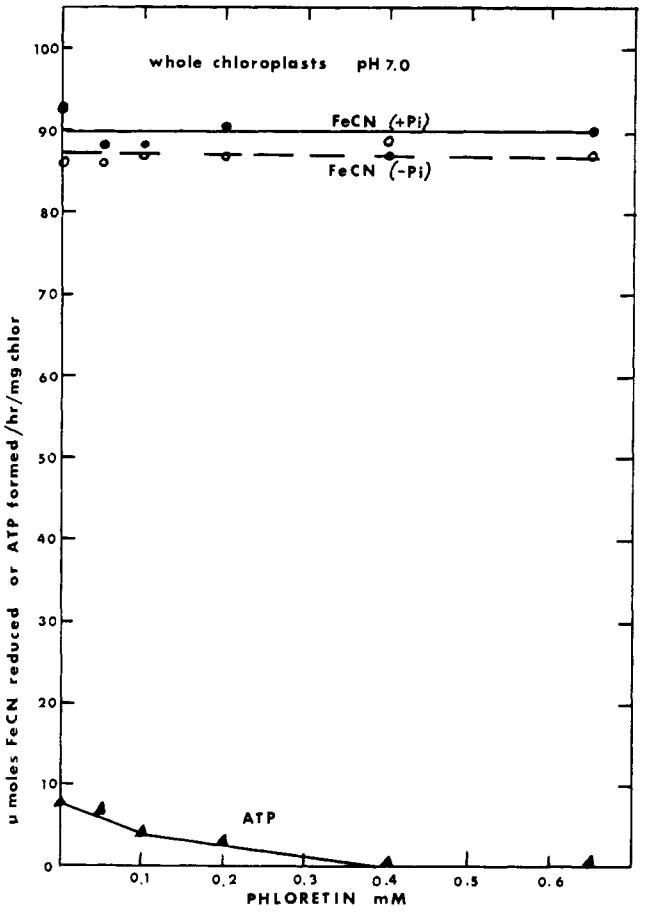

FIGURE 4: Effect of phloretin on coupled and basal electron flow at pH 7.0. Conditions as for Figure 2. Tricine (0.2 M) containing 0.2 M maleate substituted as the buffering medium.

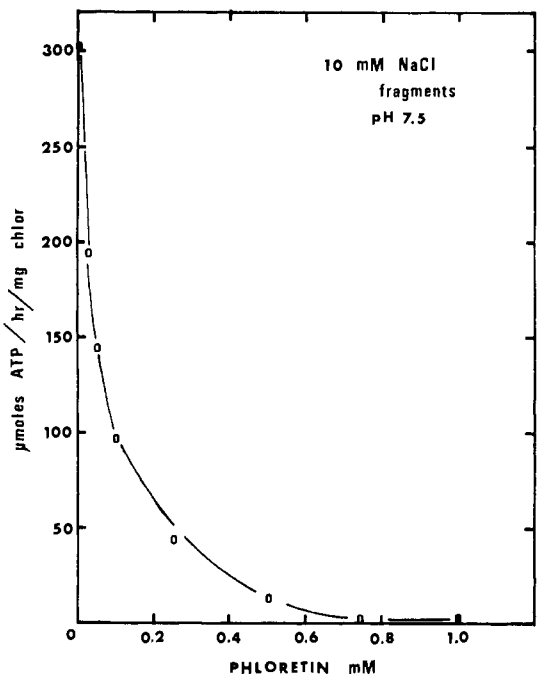

FIGURE 5: Effect of phloretin on cyclic photophosphorylation at pH 7.5. Reactions of 2.0-ml volume contained in μmoles, Tricine, pH 7.5, 80; $MgCl_2$, 10; $NaKHPO_4$, 10; ADP, 6; pyocyanine, 0.07; and [^{32}P]P_i containing approximately 5×10^5 cpm. Each reaction contained chloroplasts having 100 μg of chlorophyll and phloretin at the concentrations indicated. Reactions were illuminated at room temperature with heat filtered white light from two tungsten lamps which provided an intensity of 5.5×10^5 ergs/cm^2 per sec at the surface of the vessels. Reactions were stopped by making to 2% with trichloroacetic acid. ATP formation was measured by the method of Avron (1960).

electron flow the effect of this inhibitor on basal electron flow is the same as that reported for other energy-transfer inhibitors at pH regimes which allow phosphorylation to occur maximally.

Phloretin, on a molar basis, is about three times as effective an inhibitor of photophosphorylation as phlorizin at either pH 8.2 or 7.5 and is as effective as the phlorizin analog 4-deoxyphlorizin reported by Winget *et al.* (1969). The alteration of the inhibition pattern as a function of pH indicates that the ionization of the free hydroxyl groups in the unsubstituted phlorizin analogs may be responsible for their inhibition of the basal electron transport at pH 8.2.

Figure 5 shows that cyclic phosphophosphorylation catalyzed by pyocyanine has the same sensitivity to phloretin as the phosphorylation associated with ferricyanide reduction. The inhibition of cyclic photophosphorylation by phloretin was found to be more severe with chloroplast fragments than with whole chloroplasts as was the case with the ferricyanide Hill reaction (Figures 2 and 3; E. Uribe, unpublished experiments).

Effect of Phloretin on Phosphate-Transfer Reactions. The preceding experiments on light-driven ATP synthesis indicated that phloretin inhibits photophosphorylation by the reduction of electron flow and also by the direct inhibition of phosphorylation. It was therefore important to assess the effect of this inhibitor on the phosphorylation reaction independent of electron flow. Jagendorf and Uribe (1966a,b) and McCarty and Racker (1966) have presented evidence that acid-induced ATP synthesis is energized by a proton gradient operating independently of electron flow and utilizing only the terminal enzymes of the phosphorylating system. The acid-induced ATP synthesis reaction can thus be used to determine the effect of phloretin on phosphate-transfer reactions of phosphorylation in a system which does not depend on electron transport.

Table II shows that phloretin is an effective inhibitor of acid-induced ATP synthesis and that its effect is the same whether the compound is present in either the acid or alkaline stages of the reaction. The concentration required to obtain 50% inhibition of the phosphorylation reaction is about twofold higher than that required to inhibit photophosphorylation and supports the idea that the inhibition of phosphorylation coupled to ferricyanide reduction at pH 8.2 is a combination of its action on electron flow and phosphorylation.

The hydrolysis of ATP by chloroplasts has been shown to be catalyzed by the membrane-bound coupling factor and indeed the antibody studies of McCarty have shown that coupling factor is immunologically identical with the Mg^{2+}- and Ca^{2+}-ATPases and the ATP–P_i-exchange enzyme of the chloroplast. Experiments assessing the action of phloretin on the chloroplast ATPase enzymes are shown in Table III.

TABLE II: Effect of Phloretin on Acid-Induced ATP Synthesis.[a]

Final Concn of Phloretin (μM)	ATP ($m\mu$moles)/mg of Chlorophyll Phloretin in Acid + Base Stages	% Control	ATP ($m\mu$moles)/mg of Chlorophyll Phloretin in Base Stage	% Control
0	121		145	
12.5	121	100	128	88
25	101	83	124	85
50	98	81		
62.5			113	78
125	78	64	94	65
250	64	53	75	52
500	0	0	0	0

[a] Reactions were run as described in experimental procedures.

The light-triggered Mg^{2+}-ATPase is strongly inhibited by phloretin and again the inhibition of this activity is most pronounced when the compound is present during a portion of the reaction sequence which depends on electron flow; in this case during both the activation (light) and hydrolysis stages of the reaction. Table III also shows that phloretin is an effective inhibitor of the Ca^{2+}-ATPase activity of the soluble coupling factor at concentrations comparable with those inhibiting the membrane-bound enzyme.

The action of this compound in the inhibition of electron flow and phosphorylation is quite similar to that exhibited by dicyclohexylcarbodiimide; thus, it seemed advisable to determine whether phloretin could interact with chloroplast membranes and alter the expression of the light-induced pH rise reaction.

TABLE III: Effect of Phloretin on Chloroplast ATPase Activities.[a]

Phloretin (μM)	Inhibition (%)		
	Light-Triggered, Membrane-Bound Mg^{2+}-ATPase		Dithiotreitol-Activated Soluble Ca^{2+}-ATPase[d]
	Phloretin Activation + Hydrolysis[b]	Phloretin Hydrolysis[c]	
0	0	0	0
5	5	0	0
10	10	0	0
25	31	10	21
50	55	36	43
100	100	90	63

[a] Reactions were run as described in experimental procedures. [b–d] The control rates were 252.6, 215.3, and 28.0 μmoles of P_i released per hr per mg of chlorophyll for reactions b, c, and d, respectively.

TABLE IV: Effect of Phloretin on the Light-Induced pH Rise Reaction of Chloroplast Fragments.[a]

Phloretin (μM)	ΔpH (units)[b]	
	pH 7.5	pH 7.0
0	0.15	0.41
10	0.14	0.41
35	0.15	
50		0.40
60	0.15	
100		0.41
210	0.15	
335	0.14	

[a] Reactions were run in a thermostated Lucite chamber at 15° with illumination provided by a Kodak projection lamp. The white light delivered after heat filtering was of 5×10^5 ergs/cm^2 per sec intensity at the interior of the reaction vessel. Reactions of 5-ml volume at the initial pH noted contained NaCl, 40 μmoles; pyocyanine, 0.5 μmole; and chloroplasts containing 250 μg of chlorophyll. pH was measured using a Corning Model 12 pH meter fitted with a Thomas 4858-L60 combination electrode. The signal was recorded on a Model 7100B Hewlett-Packard recorder. [b] Figures are corrected by titration with NaOH for buffering provided by the increasing concentrations of phloretin.

In Table IV it is seen that phloretin has no effect on the light-induced proton pump at pH 7.0. Phloretin is also without effect on the proton pump at pH 7.5 at those concentrations which will significantly inhibit both electron flow and phosphorylation (Figure 2). Experiments in progress on the reconstitution of the light-induced pH rise in EDTA-treated chloroplasts have shown that while phloretin does not effect the reconstitution of the proton pump in EDTA-treated chloroplasts as does dicyclohexylcarbodiimide, it can interact with the membranes of EDTA-treated chloroplasts to alter their reconstitution by dicyclohexylcarbodiimide. It has been possible to prepare chloroplasts fragments which cannot catalyze the pH rise reaction and which can be partially reconstituted by the addition of phloretin (E. G. Uribe, unpublished experiments). These experiments will be reported in another communication.

Discussion

The experiments reported in this communication support and extend the concept of the function of compounds which inhibit energy transfer in chloroplasts. The function of such compounds as described by Izawa *et al.* (1966) was the specific inhibition of that electron flow coupled to phosphorylation and this initial demonstration of energy-transfer inhibition was followed by the description of several other

compounds with similar action (McCarty *et al.*, 1965; Gross *et al.*, 1968). Other reports have noted that certain compounds show a concentration-dependent inhibition of phosphorylation and electron flow. Octylguanidine (Gross *et al.*, 1968) and dicyclohexylcarbodiimide (McCarty and Racker, 1967) have been reported to act as energy-transfer inhibitors at low concentrations and as uncouplers at higher levels.

The experiments reported herein describe the twofold action of phloretin with the compound inhibiting phosphorylation and ferricyanide reduction in a coordinate manner at concentrations to about 50 μM (Table I). At higher concentrations the inhibition of phosphorylation is more severe and the ratio of phosphate esterified to pairs of electrons transported approaches zero with the residual electron flow provided by an inhibited basal rate. The experiments on the effect of phloretin on acid-induced ATP synthesis and the hydrolysis of ATP by the Mg^{2+}- and the Ca^{2+}-dependent ATPase enzymes (Tables II and III) show that this compound exerts dual effects on the energy conversion process by inhibiting transphosphorylation and electron flow at pH 8.2. Dio-9 and dicyclohexylcarbodiimide (McCarty *et al.*, 1965; McCarty and Racker, 1967) have been shown to inhibit the phosphate-transfer reactions of CF_1 at those concentrations which inhibit phosphorylation and electron flow in a coordinate manner. The inhibition of transphosphorylation by phloretin is thus consistent with the action of the other energy-transfer inhibitors on this reaction (McCarty and Racker, 1966, 1967) and their action as inferred by their effect on coupled electron flow (Izawa *et al.*, 1966; Gross *et al.*, 1968; Winget *et al.*, 1969). These experiments also show that the glucose moiety of the phlorizin analogs is not essential to the inhibition of the transphosphorylation reaction.

The effect of phloretin on coupled, basal, and uncoupled electron transport is unusual in that it shows a marked pH dependence. The inhibition of ferricyanide reduction in these reactions becomes progressively less severe as the pH of the reaction is lowered. At pH 7.5 (Figure 2) coupled ferricyanide reduction by whole chloroplasts is inhibited to a concentration of 0.4 mM and at this maximal inhibition of electron flow the rate is identical with the basal rate. Increasing concentrations produce no further inhibition of electron flow while phosphorylation is progressively inhibited to essentially complete inhibition at 0.65 mM. When the reactions are carried out at pH 7.0 (Figure 4) the phosphorylation reaction is essentially eliminated and phloretin is completely without effect in either the basal or coupled system while the very slight residual ATP synthesis is inhibited as at the higher pH regimes. These results show that the effect of phloretin as an energy-transfer inhibitor is clearly distinct from its effect on basal electron flow. The effect on the phosphorylation reaction and coupled electron flow is noted at all hydrogen ion concentrations at which ATP synthesis can occur while the inhibition of the basal reaction is progressively relieved by lowering the pH at which the reaction is carried out.

The differential effect of phloretin on ferricyanide reduction as a function of pH can be correlated with the results of Winget *et al.* (1969) which show that phlorizin analogs which inhibit only coupled electron flow at pH 8.0 have one glycosylated hydroxyl group *ortho* and an unsubstituted hydroxyl group also *ortho* to the carbonyl group while those analogs which have two unsubstituted hydroxyl groups *ortho* to the carbonyl function are found to inhibit both coupled and basal electron transport. It seems likely, therefore, that the inhibition of the basal electron flow at pH values maximal for phosphorylation (pH 8–8.5) is due to the action of an ionized species of phloretin in the unsubstituted phlorizin analogs. As the ionization is reduced by lowering the pH of the reaction the effect of these inhibitors becomes more similar to that noted for phlorizin at pH 8.0 indicating that the ionized compound is able to inhibit the transphosphorylation reaction effectively as the glycosylated compounds but that it also can interact with the membrane-bound electron chain while the glycosylated (un-ionized) compounds can only interact with the membrane localized phosphorylating enzymes.

The lack of inhibition of the ferricyanide Hill reaction at pH 7.0 (Figure 4) irrespective of the presence of phosphate provides further evidence that energy-transfer inhibitors as originally defined block only that portion of the electron flow which requires the enzymatic function of the coupling protein as a transphosphorylating enzyme (McCarty and Racker, 1966, 1967). The immunological and inhibitor studies of McCarty and Racker (1966) have demonstrated that nonphosphorylating energy conversion reactions such as the proton pump and ATPase activation are independent of inhibition by an antibody to CF_1 and Dio-9, thus the absence of phloretin inhibition of electron flow at hydrogen ion concentrations which prevent phosphorylation and promote maximal pH rise supports the concept that energy coupling into the proton pump is entirely independent of the enzymatic function of CF_1 in phosphorylation. This implies that CF_1 may play an indirect role in the maintenance of membrane structure and this is reinforced by the fact that CF_1 is an obligate component of the light-induced uptake of protons by chloroplasts (McCarty and Racker, 1966). The work of Uribe and Jagendorf (1968) has shown that the coupling factor is not structurally essential to the membrane in an osmotic sense; thus, it may be required to catalyze the formation or maintain the presence of a high-energy intermediate essential to the uptake of protons.

The results of these experiments may be relevant to the question of the relationship of the basal electron flow which can energize proton uptake to that which is obligately coupled to phosphorylation. One possible relationship is that discussed by Izawa and Good (1968) which regards coupled and basal electron flow as inherently different with basal flow being independent of phosphorylation. The added electron transport seen on including ADP, P_i, and Mg^{2+} in Hill reactions is therefore superimposed over a constant basal rate. This would imply that the proton pump can be energized by the basal electron flow and proton uptake would occur at all pH ranges at which electron flow can occur, as is, in fact, observed (Table IV; E. Uribe, unpublished experiments; Dilley, 1966; Schwartz, 1968). The other alternative is that the proton-uptake reaction is the primary act of energy conservation (Mitchell, 1966) and that the utilization of the energy of the pH gradient is dependent on the presence of pH regimes favorable to the enzymatic function of the coupling factor in phosphorylation. The finding that phloretin does not inhibit the proton pump at pH 7.5 at concentrations which inhibit phosphorylation 88% and coupled electron flow 23% (Figure 2) supports the idea that basal and coupled

electron flow are functionally separate pathways with differential sensitivity to phloretin.

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Properties of the Active Sites of Antibodies Specific for Folic Acid*

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ABSTRACT: Certain pteridine haptens undergo characteristic shifts in their absorbance spectra when bound by rabbit antifolate antibodies. These shifts are closely reproduced when the same haptens form complexes with L-tryptophan. Folic acid undergoes a similar spectral shift when bound by chicken liver dihydrofolic reductase. Specific inactivation of rabbit antifolate molecules by *N*-bromosuccinimide can be prevented by hapten protection of the antibody sites suggesting, together with the spectral shift of bound ligands that rabbit antibodies specific for folic acid contain tryptophan residues in their active sites. There was no significant increase in affinity or in cross-reactivity between the antifolate antibodies isolated 48 days after immunization and those obtained after a booster injection 7 months after primary immunization.

Evidence presented previously (Little and Eisen, 1967; Little and Donahue, 1968; Little *et al.*, 1969) suggests that one or more tryptophan residues forms a part of the active sites of antibodies to polynitrobenzene determinant groups. This evidence was derived primarily from the characteristic ligand spectral shifts observed on binding and the qualitatively and quantitatively similar spectra produced when the same ligands form complexes with L-tryptophan as the free amino acid. It has also been observed that the polynitrobenzenes have a high affinity for electrons while tryptophan, of all the naturally occurring amino acid residues, has the greatest ability to serve as an electron donor. Therefore, it has been proposed (Little and Eisen, 1967) that charge transfer complex formation may account for the red shifts in the absorbance spectra of DNP and TNP ligands bound in the antibody combining sites. In order to examine this possibility, we have studied here the reactions between folic acid and antifolate antibodies.

Folic acid was chosen as a haptenic determinant because it is a good electron acceptor and readily forms complexes with a variety of electron donors (Fujimori, 1959). The availability of a large number of folate analogs also provided an opportunity to examine the antibody combining sites by comparison of their affinity for a homologous series of ligands. Specifically, the following study was addressed to

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