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Inhibition of Photophosphorylation by Kaempferol[1]

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ABSTRACT

Kaempferol, a naturally occurring flavonol, inhibited coupled electron transport and both cyclic and noncyclic photophosphorylation in isolated pea (*Pisum sativum*) chloroplasts. Over a concentration range which gave marked inhibition of ATP synthesis, there was no effect on basal or uncoupled electron flow or light-induced proton accumulation by isolated thylakoids. It is suggested that kaempferol acts as an energy transfer inhibitor.

Flavonoids are a major class of secondary plant metabolites which have, as yet, an undefined role in plant metabolism. A possible site of metabolic control for which flavonoids have been suggested to have an effect is at the level of energy transduction processes. Curvature of pea tendrils, and energy-requiring process which may utilize a cellular ATPase, is inhibited by exogenous flavonoids (5). In addition, Stenlid (15) has shown that active ion accumulation by roots is inhibited by exogenous flavonoids. The latter effect was correlated to an uncoupling effect on isolated mitochondria by various flavonoids (16–18).

There have been no reports describing the effects of flavonoids on the reactions of chloroplasts, either *in vivo* or *in vitro*. Since kaempferol is a common naturally occurring flavonoid (3, 5), we have studied its effects on reactions of isolated pea chloroplasts. We will demonstrate that kaempferol is a potent inhibitor of photophosphorylation at concentrations which are physiologically significant.

MATERIALS AND METHODS

Chloroplasts were isolated from 10- to 14-day-old pea leaves, *Pisum sativum* var. Alaska. Ten grams of leaf and stem tissue were homogenized for 5 sec in a Waring Microblender in 20 ml of grinding solution (0.4 M sorbitol; 0.1 M Na-Tricine, pH 7.8; 0.07 M sodium ascorbate; and 2 mg/ml of bovine serum albumin). The brei was filtered through 4 and then 12 layers of cheesecloth and centrifuged at 1000*g* for 10 min. The resulting chloroplast pellet was dispersed in 1 ml of resuspension mix (0.4 M sorbitol; 0.05 M Na-Tricine, pH 7.8; 0.01 M KCl; and bovine serum albumin, 5 mg/ml). Chl concentration was determined according to Arnon (1).

Light-induced noncyclic electron transport, in the presence of methyl viologen (7), was monitored with a YSI model 53 oxygen monitor using a Clark electrode in a temperature-regulated flask at 20 C. Photophosphorylation was determined titrametrically by the procedure of Nishimura and Chance (13). A yellow Corning filter (3-68) was placed between the light source and sample to eliminate artifactual responses in both O_2 and pH measurements.

Methods for the activation and assay of Mg^{2+}-dependent ATPase in chloroplasts were adapted from those of McCarty and Racker (12). In a final volume of 1.0 ml, chloroplasts containing 0.1 mg of Chl were incubated for 2 min either in the dark or in intense white light at 20 C. The incubation mixture contained 50 mM Na-Tricine at pH 8.0, 25 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM DTE,[4] and 0.05 mM PMS. For assay, 0.5 ml of solution (containing 50 mM Na-Tricine at pH 8.0, 10 mM $MgCl_2$, and 8 mM ATP plus the appropriate concentration of inhibitor) was added to the reaction mix and incubated in the dark at 38 C for various time intervals. Reactions were terminated by the addition of 0.15 ml of 20% trichloroacetic acid. Phosphate release was determined by the colorimetric procedure of Rockstein and Herron (14).

Kaempferol was purchased from the Sigma Chemical Co., St. Louis, and stored as a 5×10^{-2} M solution in methanol prior to use.

RESULTS AND DISCUSSION

Noncyclic photophosphorylation, in the presence of MV, was found to be inhibited by kaempferol (Fig. 1); approximately 50% inhibition occurred at 3×10^{-5} M. It is well established that electron transport-dependent phosphorylation can be inhibited via at least three different mechanisms: by inhibiting electron transport (e^-t. inhibitors), by uncoupling the process of phosphorylation from electron transport (uncouplers), or by interfering with the terminal reactions of ATP synthesis (energy transfer inhibitors) (6, 20). We examined the effects of kaempferol on noncyclic electron transport reactions (Fig. 1). The compound had negligible effects on either basal electron flow, in the absence of ADP, or uncoupled electron transport, in the presence of 10 mM NH_4Cl. Electron transport in a complete phosphorylating reaction mix (with ADP and Pi) was inhibited, however. High concentrations of kaempferol, which reduced rates of ATP synthesis by more than 75%, inhibited coupled electron transport nearly to the level of basal electron flow (Fig. 1). These data suggest an inhibition by kaempferol of the utilization of a high energy intermediate formed during coupled electron flow. The data are consistent with previously reported effects of "energy transfer inhibitors" on electron transport and phosphorylation reactions (6, 7, 10, 11, 19, 20).

Kaempferol was also found to inhibit photophosphorylation

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[4] Abbreviations: DTE: dithioerythritol; MV: methyl viologen; PMS: phenazine methylsulfate.

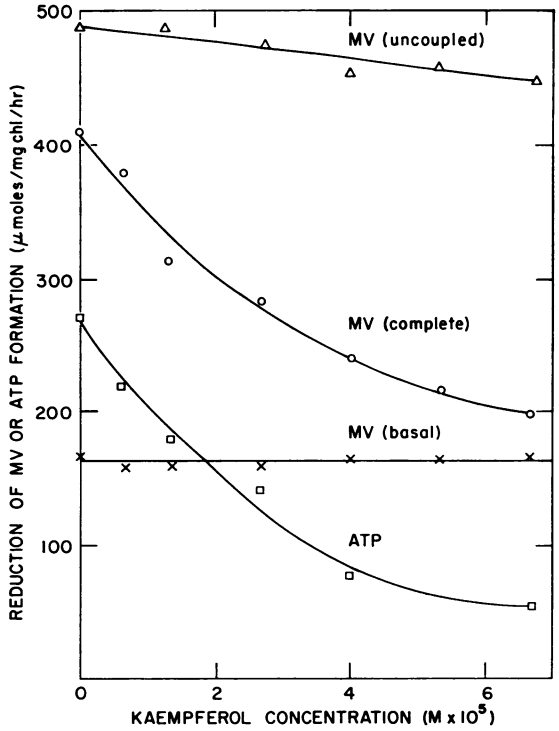

FIG. 1. The effect of various kaempferol concentrations on rates of electron transport and noncyclic photophosphorylation. The reaction mix for measurement of phosphorylation rates contained the following (in μmoles) in a total volume of 6 ml: NaN_3, 5; MV, 5; KCl, 100; $MgCl_2$, 20; sodium phosphate, 20; ADP, 8. The reaction was run at pH 7.8 at 20 C. The reaction mix for measurement of noncyclic electron flow (complete) was the same as above. For determination of basal rates of electron transport, ADP was omitted. For uncoupled rates of electron transport, NH_4Cl (final concentration, 10 mM) was added to the complete reaction mix.

in the presence of PMS (Fig. 2). Approximately 50% inhibition of ATP synthesis occurred at a flavonoid concentration of 2.5×10^{-5} M, which is in close agreement with the previous data for noncyclic phosphorylation. Since the effects of kaempferol on electron transport did not include an uncoupling action, it might be expected that the formation of light-induced high energy intermediates utilized during ATP synthesis would not be inhibited. Numerous studies have indicated that proton accumulation by chloroplast lamellae is intimately associated with the formation of such high energy intermediates (8, 9). We have therefore examined both the initial rate and total extent of proton transport into chloroplast thylakoids over a range of kaempferol concentrations. These assays were conducted at the same pH and under the same conditions which were employed for phosphorylation assays (except that arsenate replaced phosphate in the assay; see Ref. 2). The flavonoid exhibited no inhibitory effects on "proton pumping" over the entire range of concentrations tested (Fig. 2).

The data described are consistent with the idea that kaempferol acts as an energy transfer inhibitor. Previous studies with other compounds (*e.g.*, phlorizin, chlorotributyltin, etc.) which are also thought to act in this fashion, have shown that these inhibitors block the activity of a membrane-bound ATPase in chloroplasts. This "coupling factor" is thought to catalyze the terminal steps in phosphorylation (10, 11, 20). In agreement with these earlier studies, 3.3×10^{-5} M chlorotributyltin and 3×10^{-3} M phlorizin were found to inhibit strongly the activity of the chloroplast ATPase (Fig. 3). In contrast, kaempferol had only a slight effect on the ATPase (Fig. 3) even at concentrations which gave nearly total inhibition of ATP synthesis (Figs. 1 and 2). This very limited inhibition of ATPase activity suggests that kaempferol does not act in a fashion directly analogous to that of the previously described energy transfer inhibitors.

If we assume that the process of coupling of electron transport and ATP synthesis occurs by a series of reactions, or

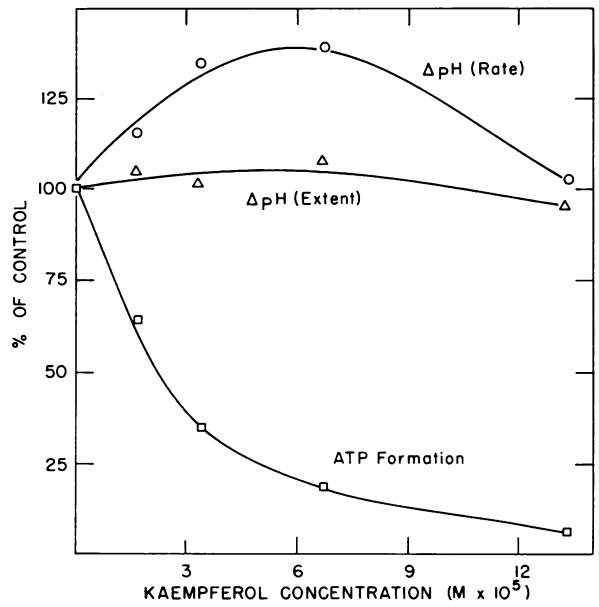

FIG. 2. The effect of kaempferol at various concentrations upon PMS-mediated H^+ transport and cyclic photophosphorylation. A 3-ml reaction mixture for cyclic photophosphorylation contained the following (in μmoles): PMS, 0.15; sodium phosphate, 10; KCl, 50; $MgCl_2$, 10; ADP, 4.5; DTE, 2; and chloroplasts equivalent to 118 μg of Chl. The reaction was run at pH 7.8 at 20 C. For measurement of proton transport, sodium arsenate (10 μmoles/3 ml) replaced sodium phosphate in otherwise identical reaction mixtures. Initial rates and maximal extent of proton transport were 325 μmoles H^+/mg Chl·hr and 0.91 μmole H^+/mg Chl, respectively. The initial rate of phosphorylation was 542 μmoles ATP/mg Chl·hr.

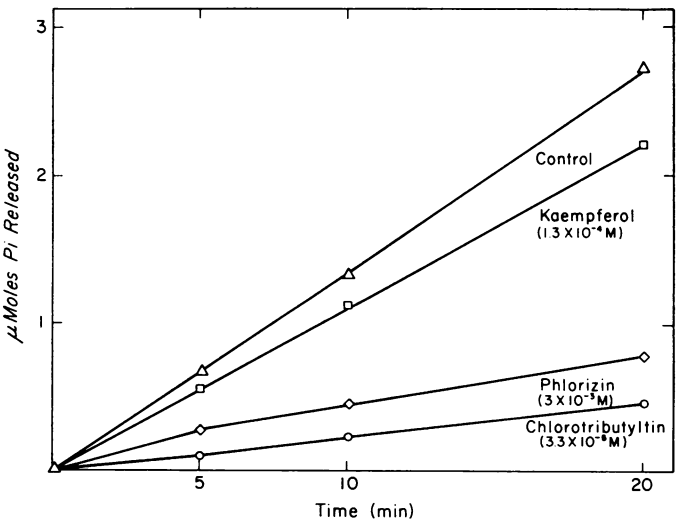

FIG. 3. Comparative effects of kaempferol, phlorizin, and chlorotributyltin on ATPase activity of isolated chloroplast lamellae.

membrane-associated processes, with proton accumulation being in equilibrium with an intermediate energized state, then we may conclude that kaempferol acts at a step subsequent to the formation of the high energy intermediate but at a site preceding the final utilization of the energy in the enzyme-mediated process of ATP synthesis. The flavonoid may therefore be useful in further elucidation of the coupling processes.

Further studies (data not shown) have indicated that quercetin, a flavonoid which is structurally related to kaempferol, acts in an identical fashion and over a very similar concentration range in the reactions described above. Preliminary studies with kaempferol or quercetin glycosides have shown very low inhibitory activity, however. The latter data may be of importance in future physiological studies of the importance of flavonoids in plant metabolism. Previous investigations with peas (3) have indicated that the flavonols, kaempferol, and quercetin occur mainly in the glycosylated form in living tissue. Furuya and Thomas (4) have reported that kaempferol-3-triglucoside and kaempferol-3-(triglucosyl-*p*-coumarate) occur at levels ranging from 1 to 4 μmoles/g fresh weight in pea seedlings. If, for the purpose of calculation, we assume a uniform distribution of the compounds in the tissue, this converts to an "average" tissue concentration of greater than 1 mM. Our data have indicated that these concentrations are at least 10-fold higher than the level of kaempferol which is required to severely inhibit chloroplast functioning. Unfortunately, these rough calculations cannot be extended with any certainty since no information is available on the cellular distribution of the flavonols within tissues or at a subcellular level. It is apparent that further analysis of localization and metabolism of flavonoids is necessary to understand their possible roles in regulating energy metabolism.

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