ABSTRACT

Kaempferol (3,5,7,4'-tetrahydroxyflavone) inhibited the rate of state 3 substrate oxidation, but not the state 4 rate. This, along with the kaempferol inhibition of substrate-driven calcium-phosphate deposition, provided evidence that kaempferol was acting specifically on the phosphorylation mechanism and not on electron transfer. Kaempferol, however, did not inhibit ATP-driven contraction while oligomycin did. Comparisons of kaempferol with mersalyl indicated that kaempferol did not inhibit phosphorylation by blocking phosphate transport. Both kaempferol and 2,4-dinitrophenol inhibited calcium-phosphate transport, but kaempferol did not stimulate respiration to the extent that 2,4-dinitrophenol did under aceptorless conditions. Kaempferol had no effect on NADH-driven contraction in a potassium chloride reaction medium. The site of kaempferol effect is thus seen to be unique from oligomycin and more like aurovertin, likely acting before the formation of the phosphorylated high energy intermediate, but not as an uncoiler in the traditional 2,4-dinitrophenol mode.

Flavonoid compounds have been reported to be distributed widely throughout the plant kingdom (11). Despite this wide distribution, little evidence has been presented linking their occurrence with a role in growth or metabolism. Indirect evidence suggesting a role in dormancy and in root and shoot growth has been presented by Harborne (11). It was determined that kaempferol (3,5,7,4'-tetrahydroxyflavone) was a cofactor for in vitro IAA oxidase while quercetin (3,5,7,3',4'-pentahydroxyflavone) inhibited the activity of the enzyme (6). Stenlid (16) conducted an extensive survey of the effects of many flavonoids on ATP production by mitochondria isolated from several plant species. From this work it is evident that minor changes in the hydroxyl pattern affect the degree of inhibition of ATP formation. The work of Stenlid (16) is valuable in that it surveys a wide range of flavonoids for effects on ATP formation. It does not, however, demonstrate the site of the flavonoid effect.

Recent experiments by Weissenbock (20) found several flavonoid glycones localized in plastids of Avena sativa L. In etiolated tissues, these flavonoids were contained primarily in the etioplasts, whereas in green leaves, the total flavonoid concentration increased by a factor of 100 and was found predominantly in the vacuole or cell wall, or both, and not in the chloroplasts (20). Kaempferol glucosides were a major component of the flavonoids quantitated.

Galston (7) has hypothesized from data with quercetin and kaempferol glucosides that flavonoids could play a broadly significant role in contractile and membranous phenomena in plants. Such possibilities would involve transport mechanisms associated with the production and utilization of high energy bonds, most probably as ATP and would thus suggest a possible interaction of mitochondria and flavonoids under certain conditions in vivo. In vitro mitochondrial experiments in which these interactions are studied should be useful in the furtherance of hypotheses such as Galston’s (7).

When the specific effects of kaempferol on mitochondrial mechanisms for oxidative phosphorylation are being considered, several sites of action are possible as evidenced in studies with other inhibitors. These include the oligomycin-sensitive site in some way associated with X ~ P (3), the DNP-sensitive site most likely associated with a proton-motive force or the nonphosphorylated intermediate (2, 8, 15), or the mersalyl-sensitive site associated with phosphate transport (4, 5, 8, 19). Additional studies of the effects of kaempferol on calcium transport and the substrate, or ATP-induced contraction of mitochondria swollen in KCl are also possible.

With this background information experiments were conducted to define more clearly the specific site of kaempferol inhibition of ATP formation as shown by Stenlid (16). Because of the common occurrence of many flavonoid compounds similar to kaempferol throughout the plant kingdom, it is hoped that more specific information detailing the effects of kaempferol on corn mitochondria might be useful in further delineating the physiological influence of flavonoids.

MATERIALS AND METHODS

Mitochondria were isolated from shoots of 3-day-old etiolated corn seedlings (Zea mays L., Wf9XM14) as previously reported (14). All experiments were performed in a 4-ml temperature-controlled (28 ± 0.2 C) glass reaction cell fitted with a Clark oxygen electrode (Yellow Springs Instrument Co.). The cell was placed in the light path of a modified Bausch and Lomb Spectronic 70 spectrophotometer. Per cent transmittance changes at 520 nm and polarographically measured oxygen uptake were simultaneously recorded on a dual channel recorder. Reaction media containing 200 mm KCl, 20 mm tris-HCl (pH 7.5), and 1 mg/ml BSA were stirred magnetically. Other additions were as given in the tables and figures. Mitochondrial protein was determined by the procedure of Lowry et al. (12). Kaempferol and oligomycin were purchased from Sigma Chemical Co.

Calcium uptake experiments were carried out utilizing the murexide (ammonium purpureate) technique described by Mela

---

1 This work was supported in part by funds from the Illinois Agricultural Experiment Station.

2 Present address: Agricultural Experiment Station, University of Idaho, Moscow, Idaho 83843.

---

Copyright © 1974 American Society of Plant Biologists. All rights reserved.
and Chance (13). Wavelengths of 540–510 were monitored in the dual wavelength mode on an Aminco DW-2 spectrophotometer. Mitochondria (approximately 1 mg of protein) were suspended in 3 ml of 0.3 M sucrose, 20 mM tris-HCl (pH 7.5), 40 μM murexide and 4 mM Pi. All calcium uptake experiments were conducted at room temperature.

RESULTS

Effects on Rates of O2 Uptake. Kaempferol (10−4 M) inhibited state 3 respiration upon addition of ADP in a manner similar to oligomycin (Fig. 1). Similarly, when 10−4 M kaempferol was added to mitochondria in state 3, there was an immediate inhibition of the state 3 rate back to the state 4 rate (Fig. 1). Inhibitions of state 3 were 40 to 80% at 5 × 10−4 M kaempferol depending on the substrate being oxidized (Fig. 2). Slight increases in this per cent inhibition were observed at higher kaempferol concentrations.

Mitochondria-oxidizing substrate in state 4, or under acceptorless conditions, only exhibited kaempferol-induced inhibitions at concentrations of 5 × 10−4 M or greater (Figs. 1, 3, and 4). At 10−4 M kaempferol inhibitions in the range of 15% were observed when succinate or malate + pyruvate were oxidized under acceptorless conditions (Fig. 4). A 6% inhibition was recorded in state 4 after 10−4 M kaempferol addition when succinate was being oxidized. In both state 4 and under acceptorless conditions, the rate of oxidation of exogenous NADH was stimulated by 10−4 M kaempferol, but inhibited at 5 × 10−4 M (Figs. 3 and 4). In all instances the 5 × 10−4 M kaempferol concentrations which inhibited substrate

**Fig. 1.** Oxygen uptake traces comparing the effects of kaempferol and oligomycin added during state 4 and state 3 respiration. The reaction media contained 200 mM KCl, 20 mM tris-HCl (pH 7.5), 1 mg/ml of BSA, 4 mM KH2PO4, and 0.7 to 0.9 mg of mitochondrial protein. Additions of 40 μmoles of malate (mal) + 40 μmoles of pyruvate (pyr), 300 μmoles of ADP (A, B, and C), 25 μg of oligomycin, or kaempferol to give a final reaction vessel concentration of 10−4 M, were made at the times indicated. In trace D 1200 nmoles of ADP were added as indicated and additions of kaempferol (final concentration 10−4 M), oligomycin (25 μg), or ethanol (control, 0.05 ml) were made at the undesignated arrow. Rates of O2 uptake are given next to the O2 uptake traces as nmoles of O2/min·mg of protein.

**Fig. 2.** Relationship of per cent state 3 inhibition and kaempferol concentration. The reaction media and concentration of additions were as given for Fig. 1. The experimental sequence was as indicated in the O2 uptake trace in Fig. 1D. The per cent inhibition is calculated from the state 3 rate before kaempferol addition and the rate after addition. State 3 rates on a nmoles O2/min·mg of protein basis were: malate + pyruvate, 41; succinate, 70; and NADH, 220. The substrates were malate + pyruvate, Δ; succinate, □; and NADH, ●.

**Fig. 3.** Relationship of per cent state 4 inhibition and kaempferol concentration. The reaction media and concentrations of additions were as given for Fig. 1. The experimental sequence was as indicated in Fig. 1 except the second ADP addition was not made. The per cent inhibition is calculated from the state 4 rate before kaempferol addition and the rate after addition. Control state 4 rates on a nmoles O2/min·mg of protein basis were: malate + pyruvate, 41; succinate, 70; and NADH, 90. The substrates were malate + pyruvate, Δ; succinate, □; and NADH, ●.
Calcium uptake by isolated mitochondria is likely driven by a high energy intermediate (9). The inhibition of this uptake (Fig. 5) suggests that the kaempferol-inhibitor site is not at the ATPase itself, but associated with a high energy intermediate. This experiment did not differentiate clearly between the effects of oligomycin, DNP, and kaempferol, although DNP and kaempferol had virtually an identical effect, whereas oligomycin was somewhat less effective.

The uptake of phosphate to produce the high energy phosphorylated intermediate is a coupled event that is inhibited by mersalyl (4, 5, 19) and can be followed spectrophotometrically as mitochondrial swelling in a sucrose-KCl reaction medium (8). This swelling was inhibited by mersalyl (8, Fig. 6), but not by kaempferol, thus suggesting that kaempferol has no effect on phosphate transport. Kaempferol, unlike mersalyl, did inhibit the active swelling after NADH addition, a result for which we have no ready explanation (Fig. 6).

**Effects on Swelling-Contraction Events.** Contraction of isolated mitochondria in the absence of Pi in a KCl reaction medium can be caused by the addition of ATP (18). Such contraction was inhibited by oligomycin, but not by kaempferol, thus suggesting that the kaempferol site was different from that of oligomycin (Table I).

In an acceptorless KCl reaction medium isolated corn mitochondria exhibit a spectrophotometrically observable contraction after substrate addition (18). Such a contraction results from the outpumping of K⁺ and is a coupled event thought to

---

**Fig. 4.** Relationship of per cent acceptorless O₂ uptake inhibition and kaempferol concentration. The reaction media without KH₂PO₄ were the same as given for Fig. 1, as were the concentrations of substrates. Substrate additions were made 2 min after the addition of mitochondria, and kaempferol was added 1.5 min later. The per cent kaempferol effect was calculated from the acceptorless rates before and after kaempferol addition. The acceptorless rates on a nmoles O₂/min·mg of protein basis were: malate + pyruvate, 30; succinate, 50; and NADH, 65.

**Fig. 5.** Effect of various inhibitors on the active uptake of calcium over a period of 5 min. Inhibitor concentrations in the base reaction media were: oligomycin, 25 μg; kaempferol, 10⁻⁴ M; and DNP, 10⁻⁴ M.

Oxidation under state 4 and acceptorless conditions were accompanied by precipitation within the reaction cell. This precipitation was not observed at the lower kaempferol concentrations which inhibited state 3 oxidation rates. It is likely, under these conditions, that the precipitation and substrate inhibition is directly related and does not represent the processes which were affected in state 3 at much lower kaempferol concentrations.

**Effects on Coupled Events.** The reduction of respiratory control to 1 (Fig. 1) suggests that the phosphorylative mechanism was in some manner inhibited by kaempferol. Since it is recognized that this mechanism for phosphorylation might be sensitive to inhibitory compounds at several sites, experiments delineating these sites were undertaken and the effects on kaempferol were compared to those of known inhibitors.
be associated with the early events of phosphorylation, possibly with the nonphosphorylated high energy intermediate (18). Table I presents evidence that substrate-driven acceptorless contraction is not affected by oligomycin, kaempferol, or DNP. Kaempferol (10^{-4} M) did increase the rate of acceptorless NADH oxidation by 38%, whereas 10^{-4} M DNP produced a 115% increase (Table I).

**DISCUSSION**

Both kaempferol and oligomycin inhibited the state 3 rates back to state 4 and did not allow any state 3 burst when added before ADP. In addition, while both compounds inhibited Ca^{2+} uptake in the presence of phosphate, kaempferol was somewhat more effective. When the effects of these compounds on ATP-induced contraction were compared, only oligomycin reduced this contraction. Thus, if ATP-induced contraction is a reversal of the process of phosphorylation as previously suggested (18), it would seem that the sites of kaempferol and oligomycin action are unique to each compound. If in a phosphorylation scheme oligomycin inhibits the formation of the high energy phosphorylated intermediate, then it appears likely that the site of kaempferol inhibition is before that of oligomycin.

Another inhibition, that could produce similar effects on state 3 to those observed with kaempferol, would be involved in phosphate transport. This transport is inhibited by mersalyl and can be followed via percentage transmittance changes (8). When comparative studies of the effects of mersalyl and kaempferol were conducted, kaempferol was found to have no effect on the transport of phosphate.

The possibility that kaempferol could be involved in the early events of energy conversion, such as those associated with NADH-induced mitochondrial contraction in KCl, was also found to be unlikely as none of the compounds tested were effective in reducing such contraction, even though the rates of O_{2} uptake were accelerated 38% by 10^{-4} M kaempferol and 115% by 10^{-4} M DNP. This is somewhat surprising since DNP has been reported to reduce the contraction of isolated corn mitochondria in KCl (10). However, in the previous experiments of Hanson et al. (10), the reaction media contained phosphate and Ca^{2+}. In studies of percentage transmission changes paralleling those of Ca^{2+} deposition presented in Figure 5, the magnitude of the contraction under these conditions closely paralleled Ca deposition within the mitochondria. Thus, the studies of Hanson et al. (10) actually parallel those of Ca^{2+} uptake presented in Figure 5 which indeed show a dramatic reduction of Ca^{2+} uptake in the presence of DNP. If the site of DNP inhibition is associated with the dissipation of I ~ X, then it seems unlikely that the pumping of K^+, which results in contraction, in a KCl medium is involved directly with I ~ X. Hanson et al. (8) suggest this schematically where they show the outpumping of K^+ to be associated with the proton motive force generated from respiration and not from the dissipation of I ~ X.

We are thus left with the site of kaempferol inhibition being somewhere between I ~ X and the oligomycin inhibitory site involved with the production of X ~ P. That this site is somewhere near that of DNP seems likely on the basis of the interaction studies where the presence of kaempferol inhibited most of the O_{2} uptake stimulation affected by DNP (unpublished results). However, it does not appear that kaempferol is analogous to DNP in its effect of dissipating the proton-motive force, since kaempferol did not produce the large stimulation of O_{2} uptake typical of the traditional DNP uncoupling. Rather, we are led to suggest that kaempferol is an inhibitor operating between I ~ X and X ~ P, but before the oligomycin-sensitive inhibitory site.

The comparison of our data with those of Connelly and Lardy (1) who found different effects of oligomycin and aurovertin on the swelling and contraction processes of rat liver mitochondria suggest that the site of aurovertin and kaempferol effect may be similar.

Our research has done little to resolve questions relating to flavonoid function in vivo. Under environmental conditions where flavonoids accumulate in vivo their function rests on two variables: (a) location within the cells, and (b) whether or not they are present as glycosides. If upon accumulation kaempferol were to be localized in the vacuole as previously suggested (17), there would be little effect on mitochondria or other membrane activities requiring ATP. However, if synthesis of flavonoids such as kaempferol is in the chloroplasts, then there must be movement of these compounds through the cytoplasm to the vacuole. If the compounds are present as glycosides, then any effect would likely be reduced (16). Stenlid (16), speculates that since sugars are introduced rather late in the biosynthesis, it is possible to have low concentrations of the aglycones in the cytoplasm. Further research on the localization of compounds such as kaempferol is clearly needed before more definitive statements can be made as to their in vivo role on energy-transfer processes.

**LITERATURE CITED**


