Effects of Rotenoids on Isolated Plant Mitochondria

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ABSTRACT

The effects of several rotenoids have been studied on potato (Solanum tuberosum L.) tuber and etiolated mung bean (Phaseolus aureus Roxb.) hypocotyl mitochondria. The selective inhibition of mitochondrial complex I is characterized by several tests: (a) no effect can be observed on exogenous NADH or succinate oxidation; (b) malate oxidation is inhibited at pH 7.5; (c) one-third decrease of ADP/O ratio appears during malate oxidation at pH 6.5 or during α-ketoglutarate, citrate, or pyruvate oxidation at a pH about 7; (d) during malate oxidation at pH 6.5, a transient inhibition appears which can be maintained by addition of exogenous oxaloacetate; (e) in potato mitochondria, the inhibition of malate oxidation disappears at pH 6.5 when NAD⁺ is added. Then, a one-third decrease of the ADP/O ratio can be measured.

Such a selective inhibition of complex I is obtained with deguelin, tephrosin, elliptone, OH-12 rotenone, and almost all the rotenoids extracted from Derris roots. The presence of the rings A, B, C, D, E seems to be necessary for the selective inhibition. Opening of the E ring and hydroxylation of the 9 position (rot-2'-enolic acid) give a rotenoid derivative with multisite inhibitory activities on flavoproteins, which are quite comparable to those of common flavonoids such as kaempferol (Ravanel et al. 1982 Plant Physiol 69: 375-378).

At the present time, almost 200 different compounds isolated from angiosperms have been classified in the isoflavonoid family (10). In contrast with the flavonoids which are widely distributed among the plant kingdom, isoflavonoids can only be found in a small number of botanical families mainly in the Leguminoseae (Lotoideae and Caesalpinioideae). Among Rosaceae, Moraceae, Amaranthaceae, Iridaceae, and Podocarpaceae, some species are able to synthesize isoflavonoids.

Originating from the C₁₅ isoflavone structure itself, some more complex molecules (rotenoids, pterocarpons, coumestans) appear in a very small number of botanical genus. Only rotenoids have been studied in this work. The rotenoid molecule (Formula I) is composed of four rings A, B, C, D; A, C, D rings characterize the C₁₅ isoflavone structure; an additional ring E is often present in rotenoids and associated with ring D. Almost 50 different rotenoids have been isolated from the genus Derris (10), Tephrosia, Millettia, Mundulea, Lonchocarpus (17), and Piscidia, which are tropical plants of the Leguminoseae family, growing in Africa, Asia, or America. For several centuries, these plants have been used to prepare hunting or fishing poisons. More recently, their insecticidal properties were discovered in Europe and several scores of tons of plant dry powder are sold every year at the present, as a major constituent of house insecticidal preparations.

Rotenoids are generally located in the superficial parts of the underground organs of the plants and often reach a very high concentration. The most common rotenoid is rotenone itself (Formula I). It has been first isolated from the roots of Robinia Nicou Aublet. This plant is commonly named ‘roten’ in Taiwan.

Formula I. Structure of the different studied flavonoids.

At present, rotenone is often used in laboratory experiments on animal or plant mitochondria (9, 11) because of its inhibitory properties on complex I. In this work, we have compared the activity of other rotenoids to that of rotenone itself, on two types of plant mitochondria in order to obtain some information on the structural features needed to obtain a specific inhibition of complex I and to determine the conditions under which the rotenoids are efficacious.

MATERIALS AND METHODS

Preparation of Mitochondria. Mitochondria from potato tubers (Solanum tuberosum L.) and etiolated mung bean (Phaseolus aureus Roxb.) hypocotyl cut from bean seedlings grown from 5 d in the dark at 26°C and 60% RH. were prepared and purified by methods previously described (8). All operations were carried out at 0 to 4°C. Following purifications, the mitochondria appeared to be virtually free from extramitochondrial contamination and had a high degree of membrane intactness, as judged by electron microscopy and by low activities of the inner membrane and matrix marker enzymes (antimycin A-sensitive NADH; Cyt c oxidoreductase and malate dehydrogenase).
addition, the mitochondria were tightly coupled: averaged ADP/O ratio for succinate was 1.8 and respiratory control ratio for the same substrate was about 3.

O₂ Uptake Measurements. O₂ uptake was followed polarographically at 25°C using a Clark-type electrode system purchased from Hansatech Ltd. (Hardwick Industrial Estate, Kings Lynn, U.K.). The reaction medium contained 0.3 mM mannitol, 5 mM MgCl₂, 10 mM KC1, 10 mM Pi buffer, 0.1% defatted BSA (medium A), and known amounts of mitochondrial proteins. Unless otherwise stated, all incubations were carried out at pH 7.2.

Uncoupling Test. An amount of intact mitochondria corresponding to 0.30 mg protein was suspended in the electrode medium containing 6 mM succinate and 300 μM ATP. After a state III-state IV transition, 10 μM carbonyl cyanide 3-chlorophenylhydrazone was added in order to inhibit the nucleotide carrier. The uncoupling effect of a substance added at this stage corresponds to an increase of the oxidation rate; 100% uncoupling effect was obtained when the rate of O₂ consumption was not further stimulated by the addition of FCCP¹ (1 μM). After each assay, the incubation medium was rapidly centrifuged at 10,000 g for 3 min (Beckman Microfuge B).

Intact mitochondria were suspended in reaction medium (NH₄Cl or NH₄NO₃ 150 mM, Tris HCl 10 mM, BSA 0.1%, pH 7.2). Passive swelling reactions were measured by changes at 540 nm in a Beckman spectrophotometer (model 25) as previously described (13). Potato mitochondria show a slow rate of swelling in NH₄⁺ salts. In these conditions a rapid passive swelling is induced by uncouplers.

Chemicals. Rotenone was purchased from Sigma. All other products were a generous gift from F. Delle Monache (Istituto di Chimica, Roma) for tefhrosin, deguelin, OH-12 rotene, and P. B. Anzeveno (Dow Chemical Laboratories) for 2-ethenyl, deguelin, rot-2'-enonic acid. All these products were dissolved in ethanol. The concentration of ethanol in the reaction medium never exceeded 3%. At this concentration, ethanol alone is almost without effect on mitochondrial respiration under our conditions (presence of BSA). Usually, 300 μM was the upper concentration of rotenoid used in our experiments. For greater concentrations, these compounds tend generally to precipitate out from the reaction medium. Rotenoid fractions of Derris root dry powder were obtained by TLC. Powder was submitted to ethanolic extraction in the dark. The crude extract was chromatographed in an atmosphere devoid of O₂ on 0.5-mm thick layers of silica gel GF 254 (Merck). The solvent used for TLC was petroleum ether, chloroform, acetone (4:1:1, v/v). Chromatograms were observed under shortwave (254 nm) UV light. Twelve UV absorbing bands were detected with increasing Rₑ from the most hydrophilic band 12 (Rₑ = 0.1) to the most lipophilic band 1 (Rₑ = 1). The bands were extracted with a small amount of ethanol and rechromatographed. After different purifications, the extract was concentrated to 200 μl. An aliquot of each fraction was cochromatographed in the presence of authentic samples in different solvent systems: dichloromethane, acetone (95:5, v/v) or chloroform, acetone, acetic acid (196:31, v/v) or petroleum ether, chloroform, acetone (4:1:1, v/v). Spectra of fractions were performed in ethanol and compared to spectra of authentic samples. Concentrations were estimated with ε of rotenone (λ max = 290 nm). We have verified that all these compounds were not oxidized or altered by the mitochondria.

¹Abbreviations: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MDH, malate dehydrogenase; ME, malic enzyme; SHAM, salicylhydroxamic acid; TNP, thionine pyrophosphate.

RESULTS

Deguelin Effects on Plant Mitochondrial Activities. (a) Coupling between Electron Transfer and Oxidative Phosphorylation. At pH 7.2, with 6 mM succinate as substrate, deguelin at concentration between 1 and 300 μM (solubility limit) does not induce any change either on the respiratory control or on the ADP/O ratio which value remains close to 1.8 (Fig. 1). Moreover, deguelin at the same concentrations, fails to show any uncoupling properties when an uncoupling test, described before (25), is employed.

In the same way, deguelin at concentrations between 1 and 300 μM does not induce swelling of mitochondria in the presence of NH₄Cl or NH₄NO₃ 0.15 mM (result not shown); deguelin is, therefore, unable to allow a transfer of protons through the mitochondrial inner membrane.

All these results together demonstrate that deguelin is ineffective on the phosphorylation mechanism, i.e. proton gradient formation, activities of the coupling factor, and of nucleotide carrier.

(b) Deguelin Effects on the Electron Transfer with Succinate or NADH (Fig. 2). Deguelin at concentrations between 1 and 300 μM is without effect on the rates of succinate or NADH oxidation in the presence of ADP or of an uncoupler such as FCCP, in potato or mung bean mitochondria. Deguelin is, therefore, without inhibitory properties either on the external NADH dehydrogenase, succinate dehydrogenase, and complex III and IV.

(c) Deguelin Effect on Malate Oxidation (Fig. 2). Malate oxidation by plant mitochondria involves the enzyme MDH and NAD⁺-linked ME, both of which are localized in the mitochondrial matrix (7). ME catalyses the oxidative decarboxylation of malate to pyruvate with NAD⁺ and Mn⁴⁺ as cofactors (15). The activity of ME in intact mitochondria is influenced by a number of factors, including the pH of the external medium (16). At higher pH (7.5 or more depending on the species), ME is less active than at lower pH. The concentration of NAD⁺ within the mitochondrial matrix can also influence the activity of ME, with higher concentrations stimulating, and this can be altered by adding NAD⁺ to isolated mitochondria (27).

MDH activity is reversible and, in the mitochondrial matrix, the characteristics of malate oxidation strongly support the evidence that the regulation in vivo of MDH activity can be readily accounted for by equilibrium effect alone (2, 20). MDH activity, on the other hand, is more active at higher pH. When oxaloacetate and NAD⁺ concentrations increase in the matrix space, MDH produces malate; MDH catalyzes the formation of oxaloacetate and reduces the concentration of NAD⁺ only when the ratios oxaloacetate:malate and NADH/NAD⁺ are low. At pH 7.5, MDH is operating alone (2, 20). Under state III conditions, NADH/NAD⁺ ratio is low and the equilibrium of the MDH reaction has moved toward oxaloacetate formation. One of the reasons why potato mitochondria are able to consume O₂ at significant rates at high pH with malate as substrate in the absence of a biochemical system to remove oxaloacetate, is because they excrete oxaloacetate into the external medium (20).

At pH 7.5 in the presence of 5 μM deguelin, O₂ uptake is inhibited (Fig. 2). This inhibition could concern complex I or MDH itself. Mitochondrial treatment with Triton X100 permits to obtain a MDH solution. The formation of NAD⁺ by the extracted enzyme, measured spectrophotometrically, in the presence of oxaloacetate and NAD⁺ is not modified by addition of deguelin between 1 and 300 μM (result not shown). Since deguelin is without effect on NADH and succinate oxidation, the inhibitory activity of deguelin observed with malate is, therefore, located at the level of complex I. As for rotenone, the strong effect of deguelin can be explained as an inhibition of the electron transfer at the level of one of the several non-haem iron centers associated with the internal NADH dehydrogenase complex I.
which is lower affinity complex the that, at pH 6.5, ME is strongly active. Addition of 1 μM deguelin induces a transient inhibition of state III rate of O₂ uptake with malate as substrate (Fig. 2). At the same time, the ADP/O value, which was near 3 with malate before addition of deguelin, decreases by one-third (Table I) indicating that the deguelin-resistant pathway is nonphosphorylating. These results suggest that, at pH 6.5 in the presence of 1 μM deguelin, as with rotenone, the complex I which possesses a high affinity for NADH, is inhibited and is bypassed by another NADH dehydrogenase, which is rotenone resistant, nonphosphorylating, and has a much lower affinity for NADH (3). The transient inhibition which appears just after injection of deguelin reflects the concerted operation of MDH and ME (23). Just after the addition of deguelin, previously accumulated oxaloacetate reenters the mitochondria and is reduced via MDH at the expense of NADH produce by ME; consequently, O₂ uptake is initially slow. When all the oxaloacetate has been removed, NADH becomes accessible to the respiratory chain again and O₂ uptake rates increase (Fig. 2). Under these conditions, the rotenone-insensitive internal NADH dehydrogenase may be engaged (18). This interpretation of biphasic inhibition by deguelin is supported by analysis of the products of malate oxidation which has shown that pyruvate accumulates while oxaloacetate levels decrease during the initial phase of inhibition (result not shown). It is interesting to note that these results obtained with deguelin at pH 6.5 concern potato
Table I. Effect of Deguelin on the ADP/O Ratio Values Observed During the Course of Substrate Oxidation by Potato Tuber Mitochondria

<table>
<thead>
<tr>
<th>Substrates: 1 mM NADH, 15 mM malate + 1 mM NAD+*, 10 mM α-ketoglutarate + 150 μM TPP + 1 mM NAD+, 5 mM pyruvate + 0.5 mM malate + 150 μM TPP + 1 mM NAD+; in these conditions, the rate of pyruvate oxidation remains sufficient (i.e. oxaloacetate delivery is sufficient), after 10 μM deguelin addition, to permit a good determination of ADP/O.</th>
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<tbody>
<tr>
<td>Substrates</td>
</tr>
<tr>
<td>NADH</td>
</tr>
<tr>
<td>Malate (pH 6.5)</td>
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<tr>
<td>α-Ketoglutarate</td>
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<td>Pyruvate</td>
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Fig. 3. Effect of deguelin on state III rates of substrate oxidation by NAD+-deficient potato mitochondria. Substrates: 1 mM NADH (pH 7.2, I = 250), 6 mM succinate + 150 μM ATP (pH 7.2, I = 185), 10 mM citrate (pH 7.2, I = 32), 10 mM α-ketoglutarate + 150 μM TPP + 2 mM malonate (pH 7.2, I = 40), 5 mM pyruvate + 0.5 mM malate + 150 μM TPP (pH 7.2, I = 37) 15 mM malate (pH 6.5, I = 29). I = Initial oxidation rate (nmol O2 consumed min⁻¹ mg⁻¹ protein). When deguelin concentration in the medium is 1 μM it correspond to 1 nmol mg⁻¹ protein. Deguelin is strongly inhibited by deguelin (Fig. 3). When NAD⁺ is added to the reaction medium, O2 consumption rate with a-ketoglutarate or citrate at pH 7.2, in the presence of deguelin, is comparable to O2 consumption obtained with malate at pH 6.5 (see Fig. 2). Nevertheless, the transient inhibition never appears (Fig. 2) with a-ketoglutarate or citrate, because no oxaloacetate is accumulated in the medium.

Effects of Rotenoids on Plant Mitochondrial Activities. Five different rotenoids have been compared: deguelin, tephrosin, elliptone, 12-OH rotenone, and rot-2’-enoic acid (Formula I). Rot-2’-enoic acid is a structure appearing in rotenoid interconversion (1). The four first above mentioned rotenoids are without effect on O2 consumption by potato or mung bean mitochondria with NADH or succinate as substrates. On the contrary, rot-2’-enoic acid induces an inhibition of the electron flow with these substrates.

Figure 4 (A and B) shows that each of the studied rotenoids severely inhibits O2 consumption when malate is the substrate at pH 7.5. A 100% inhibition is obtained with 2 to 5 μM deguelin; however, rotenone, OH-12 rotenone, tephrosin, and elliptone are slightly less potent inhibitors, either with mung bean hypocotyl or potato tuber mitochondria. Rot-2’-enoic acid is much less effective than the other rotenoids; a 100% inhibition is obtained at a concentration higher than 300 μM.

In mitochondria where endogenous NAD⁺ concentration is not a limiting factor (mung bean mitochondria or potato mitochondria supplemented with exogenous NAD⁺), at pH 6.5 with malate as substrate, each of the studied rotenoids induces a transient inhibition of state III rate of O2 uptake (Fig. 5). Again, after addition of each of the studied rotenoids (except for rot-2’-enoic acid), ADP/O ratio values decrease 1 unit (Table II). The rotenoid insensitive pathway which is operating at this stage can be fully inhibited by antimycin A. When endogenous NAD⁺ concentration is low—aged potato mitochondria (21)—at pH 6.5 with malate as substrate, all of these rotenoids strongly inhibit O2 uptake, as at pH 7.5. Whereas rotenone, elliptone, deguelin, tephrosin, and OH-12 rotenone appear to be quite comparable selective inhibitors of complex I, rot-2’-enoic acid has a particular behaviour: its inhibitory efficiency on complex I is lower; 100 μM are needed for a 70 to 85% inhibition of malate oxidation at pH 7.5. Moreover, it has a non-negligible inhibitory action on NADH or succinate oxidation (40 and 25%, respectively at saturation). In addition, this compound, in contrast with the other studied rotenoids, exhibits an uncoupling effect. This effect appears at a concentration of 250 μM. Its intensity represents 95% of the FCCP effect in potato mitochondria and 90% in mung bean mitochondria. As a whole, rot-2’-enoic acid can be classified among the inhibitory uncouplers (25).

Effects of Different Flavonoids Isolated from Derris Root Powder on Mitochondrial Activities. It was interesting to compare the pure rotenoids studied here with the whole flavonoid mixture present in the roots of one of the best source of rotenoids, the genus Derris. For this purpose, Derris root dry powder was submitted to ethanolic extraction in the dark, at laboratory temperature, in an atmosphere devoid of O2. Twelve different UV₃₆₅ absorbing bands were obtained after TLC and were purified by rechromatography with the same procedure. TLC in different solvents in the presence of test substances and UV-visible spectral analysis allowed the identification of rotenone and deguelin in the band 6, of elliptone (band 7) and of 12-OH rotenone (band 8). Tephrosin seemed not to be present in the studied extract of Derris root powder. After purification, the compounds isolated from each of the 12 bands were dissolved in ethanol, submitted to spectrophotometric quantitative estimation and their effects on plant mitochondria were studied.

At concentrations lower than 50 μM none of the studied...
compounds inhibits NADH or succinate oxidation. On the contrary, every compound strongly inhibits malate oxidation at pH 7.5 (Fig. 6). This inhibition is greater on potato than on mung bean mitochondria. $I_{50}$ (concentration giving a 50% inhibition of the electron flow) measurements at pH 7.5 with malate as substrate allows a classification of the different compounds: (a) compounds corresponding to bands 5, 6, 8 give an $I_{50}$ between 0.3 and 1.5 $\mu$M. These compounds are as good or better inhibitors as rotenone itself. (b) Compounds corresponding to bands 1, 3, 9, 7 give an $I_{50}$ between 1.5 and 4.5 $\mu$M; (c) compounds corresponding to bands 4, 10, 11, 12 give an $I_{50}$ between 5 and 10 $\mu$M. When the lipophilic character of these flavonoids decreases (bands 10, 11, 12) their inhibitory potency decreases slightly. At pH 6.5, with malate as substrate in the presence of nonlimiting concentration of NAD$,^+$ the compounds corresponding to bands 5, 6, 7, 8, 9 induce a transient inhibition which is followed by a marked increase of the $O_2$ consumption. At this stage, a decrease of 1 unit of the ADP/O ratio can be shown (Table II).

**DISCUSSION**

The experimental procedures employed here permit the characterization of products inhibiting selectively complex I. These tests derive from about 30 years of scientific research which has progressively explained the mode of action of compounds such as rotenone, barbiturates, amytal, or piericidin A on plant or animal mitochondria.

In plant mitochondria from several botanical species, the specific inhibition of malate oxidation by rotenone has been studied by Chauveau and Lance (6), Palmer (22), Wilson and Hanson (28), Wiskish and Day (29). Our results demonstrate that under state III conditions deguelin and various rotenoids strongly inhibit the oxidation of all NAD$^+$-linked substrates insofar as the total amount of mitochondrial NAD$^+$ is low. In the presence of NAD$^+$, only the oxidation of malate in state III is rotenone sensitive. The degree of malate inhibition induced by deguelin is also linked to the concentration of oxaloacetate.

**FIG. 4.** Effects of various rotenoids on malate oxidation by potato tuber (A) and mung bean hypocotyls (B) mitochondria. Dotted lines show the inhibition with the rotenoids reference: rotenone. Substrate: 15 mM malate + 1 mM NAD$^+$ (pH 7.5).
It has previously been observed that the ADP/O ratio with NAD*-linked substrates was decreased by one-third in the presence of rotenone (3). The same thing holds with deguelin and various rotenoids (Table II). These results indicate that the deguelin-resistant pathway is nonphosphorylating and that deguelin like rotenoids engage the functioning of an internal rotenone-insensitive NADH dehydrogenase which is only active under conditions where the matrix concentration of NADH is high (i.e. in mung bean hypocotyl mitochondria or NAD*-supplemented potato tuber mitochondria).

The results obtained with rotenone have led us to define better the tests needed to assess that a compound is a selective inhibitor of complex I: (a) no effect on NADH or succinate oxidation; (b) inhibition of malate oxidation at pH 7.5; (c) one-third decrease of the ADP/O ratio during malate oxidation at pH 6.5 or α-ketoglutarate, citrate or pyruvate oxidation at a pH about 7; (d) during malate oxidation at pH 6.5, transient inhibition which can be maintained by addition of exogenous oxaloacetate; (e) in NAD*-deficient mitochondria disappearance of the inhibition of malate oxidation at pH 6.5 when NAD* is added. Then a one-third decrease of the ADP/O can be measured.

We have shown that all the rotenoids tested here (with the exception of rot-2'-enoic acid) are acting in the same way and are, therefore, selective inhibitors of the complex I. Deguelin and probably the compound corresponding to band 8 from Derris roots are more potent inhibitors than rotenone itself. When the rings A, B, C, D, E of the rotenoid structure are present in the formula, structural details concerning the E ring seem not to change the selective inhibition of complex I. Furthermore, the 6',8' double bond present in rotenone itself does not play an important role for the inhibitory potency. In contrast with Johnson-Flanagan and Spencer (12), the effects of elliptone are similar to those observed with rotenone and we have never observed any conversion of rotenone to elliptone in the reaction medium.

Replacement of —O by —OH on the 12 position or suppression of the lateral chains on the 5' position induce a slight decrease of the selective inhibitory activity. A comparable result is obtained when a —OH is added on the 12-a position. In marked contrast, opening of the E ring induces a great loss of the inhibitory potency (case of the rot-2'-enoic acid). In the same way, suppression of the B ring (case of the isoflavone osajin: formula I, result not shown) gives products without selective inhibitory action on complex I. On the whole, our results show that the presence of rings A, B, C, D, and E is necessary to obtain

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### Table II. Effect of Various Rotenoids on the ADP/O Ratio Values Observed During the Course of Malate Oxidation by Potato Tuber and Mung Bean Hypocotyl Mitochondria

<table>
<thead>
<tr>
<th>Substrates: 15 mM malate + 1 mM NAD* (pH 6.5).</th>
<th>Potato</th>
<th>Mung Bean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (μM)</td>
<td>ADP/O</td>
<td>ADP/O after rotenoid addition</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>--------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Rotenone</td>
<td>5</td>
<td>2.7</td>
</tr>
<tr>
<td>Deguelin</td>
<td>2</td>
<td>2.6</td>
</tr>
<tr>
<td>12-OH rotenone</td>
<td>20</td>
<td>2.4</td>
</tr>
<tr>
<td>Tephrasin</td>
<td>50</td>
<td>2.4</td>
</tr>
<tr>
<td>Elliptone</td>
<td>50</td>
<td>2.8</td>
</tr>
<tr>
<td>Rot-2'-enoic acid</td>
<td>50</td>
<td>2.7</td>
</tr>
<tr>
<td>TLC band 5</td>
<td>5</td>
<td>2.3</td>
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<tr>
<td>TLC band 6</td>
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<tr>
<td>TLC band 7</td>
<td>10</td>
<td>2.3</td>
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<tr>
<td>TLC band 8</td>
<td>10</td>
<td>2.3</td>
</tr>
<tr>
<td>TLC band 9</td>
<td>10</td>
<td>2.3</td>
</tr>
</tbody>
</table>

FIG. 5. Effect of various rotenoids on state III rate of malate oxidation by potato tuber mitochondria. The oxidation of 15 mM malate + 1 mM NAD, pH 6.5 was measured as O2 consumption in medium A (see "Material and Methods") with 0.30 mg mitochondrial protein ml-1. ADP (1 mM), rotenoids, and antimycin A (5 μM) were added where indicated. Numbers on traces refer to nmol O2 consumed/min·mg protein.
the selective inhibition of complex I. It seems that all rotenoids presenting these five rings in their structure will be selective inhibitors of complex I, with a more or less good efficiency, following the substitutions. We are not able to estimate the role played by substitutions on ring A because all the analyzed rotenoids were substituted in the same way by —OCH$_3$ on the second and third positions. It is interesting to compare our results with those of Burgos and Redfearn (4) on the effect of various rotenoids on isolated complex I from rat liver mitochondria. They demonstrate that without the E ring, or when the B or C rings are open, rotenoids are weakly active on purified complex I. The complex I of animal mitochondria seems to be much more sensitive to rotenone than that of plant mitochondria.

Our work on rot-2'-enolic acid shows a loss of selective activity on complex I. This compound has inhibitory properties on the mitochondrial electron transfer which seems to be located in the flavoprotein region. Such medium multisite inhibition can be obtained with a great number of other flavonoids like kaempferol (26) or with a great variety of other compounds. Such effects could perhaps be explained by a loss of inner membrane fluidity, induced by all these substances (19). Nevertheless, we have shown (14, 24, 26) that such multisite inhibitions do not have exactly the same characters according to the different compounds tested. It is interesting to note that uncoupling properties appear in the rotenoid group only with rot-2'-enolic acid. Opening of the E ring and hydroxylation of the 9 position seem to participate to the induction of this new property. Free hydroxyls present in tephrosin and OH-12 rotenone are unable to permit uncoupling.

Our results show that, in *Derris* root, not rotenone alone but all the complex pattern of rotenoids is active on mitochondrial complex I. These rotenoids are present in the plant as free aglycones, reaching very high concentrations in the root. It is, therefore, possible that the regions accumulating rotenoids could be anatomically or physiologically isolated from the cytoplasm. In the *Derris* root, the significance of such a high rotenoid accumulation seems evidently to be ecophysiological. Rotenoids are certainly toxins preventing predation or parasitism on the root by animals, especially invertebrates.

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