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# Action and Structure - Activity Relationship of Rotenoids as Inhibitors of Respiration *In Vitro*

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Rotenoids are plant products and are used as insect and fish poisons (CROMBIE 1963). There has been renewed interest in studies on plant products as selective insecticides (MANGAL NATH 1978, MEINWALD et al. 1978) because the commonly employed organo-phosphorus and organo-chlorine synthetic insecticides are implicated as hazardous environmental pollutants. Rotenone (1) inhibits respiration by blocking the reduced nicotinamide adenine dinucleotide (NADH) - dehydrogenase segment of the respriratory chain. Thus oxygen uptake by pyruvate or glutamate is inhibited while the oxidation of succinate wherein NADH is bypassed remains unaffected with rotenone (LINDAHL and OBERG 1961, ERNSTER et al. 1963). A report on the structure-activity relationship of rotenoids (BURGOS and REDFEARN 1965) mentions that rotenoids having a dimethyl chromene system (7) as E ring (D/E rings angularly fused) have activities as NADH oxidase inhibitors comparable with that of rotenone. No literature is available on the activity of rotenoids having D/E linear fusion. In this communication the structure-activity relationship and mode of action of rotenoids, having D/E linear fusion and incorporating a furan ring as ring E, is reported.

## MATERIALS AND METHODS

Wistar strain male rats weighing 100-120 g were used for preparing liver slices and those weighing 150-200 g were used for preparation of liver mitochondria. Slices were prepared from fresh liver by conventional free hand slicing (UMBREIT et al. 1972). Each Warburg flask contained 2.95 ml of Krebs-Ringer phosphate, pH 7.4, containing 1 mg glucose per ml of solution, rotenoid dissolved in 0.05 ml absolute alcohol and about 50 mg (wet weight) of liver slices in the main compartment. Control flasks contained no rotenoid. The central well contained 0.1 ml of 40 per cent KOH. The flasks were

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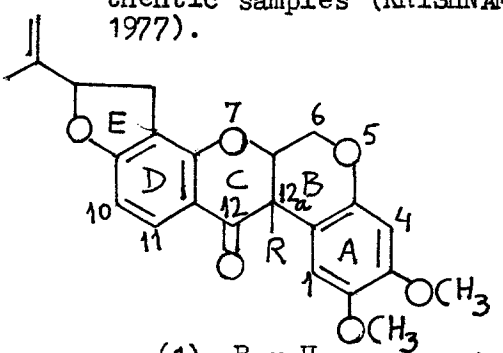
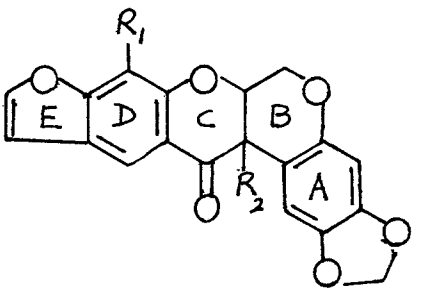
allowed to equilibrate thermally and the respiration was measured for 30 minutes. Slices were then removed, blotted and dried to constant weight in stainless steel planchets at 105 ± 1°C.

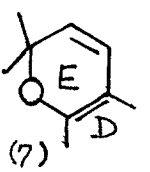
Mitochondria were isolated by the method of JOHNSON & LARDY (1967). The mitochondrial pellet was washed twice with 0.25 M sucrose and finally suspended in 0.25 M sucrose, so that 1 ml of suspension contained about 10-14 mg of protein. Mitochondrial respiration and phosphorylation were measured manometrically by the method of UMBREIT et al. (1972). (details given in Table II). Tightly coupled mitochondria giving P/O ratio of 1.7 - 1.9 and 2.6 - 2.8 for succinate and glutamate respectively were used in the experiments. After measuring the oxygen uptake for 25 minutes, the flasks were quickly removed, chilled in ice and the reaction stopped by addition of 1 ml of 10% ice-cold trichloroacetic acid. In both control and experimental flasks, inorganic phosphate in the supernatant was estimated by the method of FISKE AND SUBBAROW (1925). Protein was precipitated with 10% trichloroacetic acid, washed twice with alcohol - ether (3:1), dried and dissolved in 0.5 ml of 1N NaOH. Aliquots of this solution were used for measurements of protein by the method of LOWRY et al. (1951). Rotenoids used in the present study were isolated from Pachyrrhizus erosus seeds (MANGAL NATH 1978, KALRA et al. 1977). Final purification of the compounds was carried out by preparative t.l.c. on silica gel 'G' plates using $CHCl_3$-benzene (1:1) for each of rotenone (1), dolineone (2) and pachyrrhizone (4). For $12_a$-hydroxy-dolineone (3) and $12_a$-hydroxypachyrrhizone (5), the plates were run twice in the same direction in $CCl_4$-ether (2:1). The purity of the compounds was ascertained by physical and chemical tests and by comparison with authentic samples (KRISHNAMURTI et al. 1970, KALRA et al. 1977).




(1), R = H
(6), R = OH




(2), $R_1$ = H, $R_2$ = H
(3), $R_1$ = H, $R_2$ = OH
(4), $R_1$ = $OCH_3$, $R_2$ = H
(5), $R_1$ = $OCH_3$, $R_2$ = OH




(7)

## RESULTS AND DISCUSSION

The effect of rotenoids on respiration by rat liver slices is presented in Table I. It is clear that at the concentrations used, respiration *in vitro* was significantly inhibited by all the rotenoids when glucose was used as the substrate. The different rotenoids showed the following order of decreasing potency:- rotenone, 12a-hydroxypachyrrhizone, pachyrrhizone, 12a-hydroxydolineone, dolineone.

Thus introduction of an extra $OCH_3$ at C-8 (pachyrrhizone over dolineone or 12a-hydroxypachyrrhizone over 12a-hydroxydolineone) resulted in enhanced respiratory inhibition. Introduction of an OH group at 12a-position also increased the inhibitor potency. The metabolism of rotenoids is essentially oxidative (Yamamoto *et al.* 1971, FUKAMI *et al.* 1967). Thus 12a-hydroxyrotenoids have a higher inhibitor potency than rotenoids on account of their slower detoxication rate YAMAMOTO *et al.* 1971). Compounds possessing a furan ring E (2-5) (D/E linear fusion) exhibited respiratory inhibitor potency comparable with that of rotenone(1) (D/E angular fusion).

TABLE I

Effect of rotenoids on respiration of rat liver slices

The main compartment of the Warburg flask contained 2.95 ml of Krebs - Ringer phosphate, pH 7.4, containing 1 mg glucose per ml and rotenoid dissolved in 0.05 ml ethyl alcohol. About 50 mg (wet weight) of liver slices were added to each flask. Respiration was followed for 30 minutes at 37°C with oxygen as gas phase. No rotenoid was present in control flasks.

| Rotenoid | Concentration (Amount added) | $O_2$ consumed ($\mu$ litres)/mg. dry tissue/hr | p values |
|---|---|---|---|
| Control | - | 7.81 ± 0.45 | - |
| Rotenone | 0.045 | 3.45 ± 0.30 | <0.05 |
| Dolineone | 0.050 | 5.80 ± 0.46 | <0.05 |
| 12a-Hydroxydolineone | 0.050 | 5.20 ± 0.35 | <0.05 |
| Pachyrrhizone | 0.055 | 4.65 ± 0.25 | <0.05 |
| 12a-hydroxypachyrrhizone | 0.055 | 3.54 ± 0.28 | <0.05 |

The values are mean ± S.E of four separate experiments. Data have been statistically evaluated and $P \leq 0.05$ is considered statistically significant.

TABLE II

Effect of Rotenoids on oxidation of succinate by rat liver Mitochondria

Mitochondrial respiration was measured manometrically. Each Warburg flask in a final volume of 3 ml contained 40 $\mu$ moles $KH_2PO_4$, pH 7.4; 30 $\mu$ moles sodium succinate pH 7.4; 15 $\mu$ moles $MgSO_4$, 6 $\mu$ moles ATP, mitochondria equivalent to 3.7 mg of protein, 250 $\mu$ moles sucrose. Rotenoids were dissolved in 0.05 ml absolute alcohol. Reaction was started by tapping in 50 $\mu$ moles of glucose and 140 units of hexokinase from the side arm after equilibration at 30°C for 10 minutes. Oxygen uptake was followed for 25 minutes.

| Rotenoid tested | $O_2$ consumed ($\mu$litres)/ mg. protein/hr and p values | | | |
|---|---|---|---|---|
| | Rotenoid concentration (amount added) $5 \times 10^{-7}$M | p values | Rotenoid concentration (amount added) $2 \times 10^{-6}$M | p values |
| Rotenone | 88.4 ± 5.4 | >0.05 | 93.0 ± 5.2 | <0.05 |
| Dolineone | 88.0 ± 3.2 | >0.05 | 91.8 ± 4.0 | <0.05 |
| $12_a$-Hydroxydolineone | 87.8 ± 4.0 | >0.05 | 80.5 ± 3.9 | >0.05 |
| Pachyrrhizone | 99.0 ± 6.5 | >0.05 | 90.8 ± 6.0 | >0.05 |
| $12_a$-Hydroxypachyrrhizone | 90.8 ± 3.6 | >0.05 | 95.7 ± 4.5 | <0.05 |
| Control | 91.7 ± 6.6 | - | 84.0 ± 6.1 | - |

The values are mean ± S.E for six separate mitochondrial preparations. Data have been statistically evaluated and $p \leq 0.05$ is considered statistically significant.

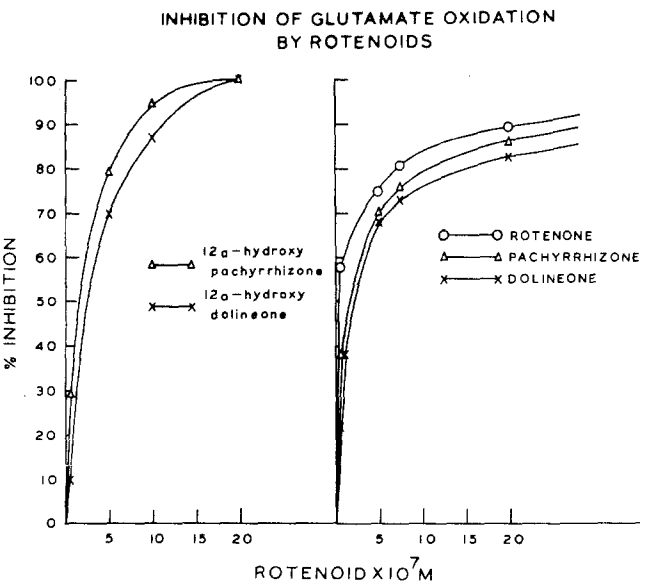
TABLE III

Effect of rotenoids on oxidation of glutamate by rat liver mitochondria

Experimental details were the same as in Table II, except for substitution of succinate by 30 $\mu$ moles of sodium glutamate.

| Rotenoid tested | Percent inhibition at Rotenoid concentration of | | | |
|---|---|---|---|---|
| | $5 \times 10^{-8}$M | $5 \times 10^{-7}$M | $1 \times 10^{-6}$M | $2 \times 10^{-6}$M |
| Rotenone | 58.00 | 75.00 | 81.00 | 89.36 |
| Dolineone | 21.80 | 68.57 | 73.20 | 82.38 |
| $12_a$-Hydroxydolineone | 10.20 | 69.92 | 87.00 | 100.00 |
| Pachyrrhizone | 38.00 | 70.20 | 76.10 | 85.87 |
| $12_a$-Hydroxypachyrrhizone | 28.50 | 79.40 | 94.50 | 100.00 |

The values are statistical average of results from six separate mitochondrial preparations.



Tables II and III show the effect of adding varying concentrations of different rotenoids to actively respiring mitochondria and studying the oxidation of succinate and glutamate respectively. Succinate oxidation was almost unaffected by rotenoids at levels of $5 \times 10^{-7}$M. However at $2 \times 10^{-6}$M concentration rotenone, dolineone and 12a-hydroxypachyrrhizone showed slight stimulating effects in agreement with earlier observation made on rotenone (LINDAHL & OBERG 1961, ERNSTER et al. 1963). Glutamate inhibition by all the rotenoids was significant, even at concentration of the order of $10^{-7}$M. Thus all rotenoids have a mode of action similar to that established for rotenone by LINDAHL and OBERG (1961). For all the rotenoids the inhibition of glutamate with increasing concentrations was found to have hyperbolic trends (Fig. I). Similar hyperbolic trends in NADH-oxidase inhibition with increasing concentration of inhibitor have been reported by BURGOS and REDFEARN (1965).

The comparative inhibition trends here are interesting and help in rationalizing the structure-activity relationship as well as mode of action of the rotenoids. At lower concentrations ($0.5 \times 10^{-7}$M) the 12a-hydroxy rotenoids are less inhibitory than their counterparts (with no 12a-hydroxy group) but at higher concentration, the increase in inhibition with 12a-hydroxyrotenoids is sharper and 100% inhibition is quickly attained. For pachyrrhizone (4), dolineone (2) and rotenone (1), initial increases in inhibition are sharper but beyond a certain level, these increases become gradual. YAMAMOTO et al (1971) observed less inhibition of NADH oxidase with 12a-hydroxyrotenone (6) than with rotenone (1) at all levels of concentration. Thus at lower concentrations, the results on 12a-hydroxypachyrrhizone (5) and 12a-hydroxydolineone (3) are in agreement with those of YAMAMOTO et al. (1971). However at higher concentrations 12a-hydroxydolineone (3) and 12a-hydroxypachyrrhizone (5) are much more inhibitory than dolineone (2) and pachyrrhizone (4) respectively. Presumably, the additional isopropenyl chain in rotenone (1) and 12a-hydroxyrotenone (6) and consequent increased liposolubility might account for this difference. Presence of bulky alkyl groups is considered to contribute to higher insecticidal activity (MURAOKA and TERADA 1972). The work of HORGAN et al. (1968, 68a) and of GUTMAN et al (1970, 1970a, 1971) has shown involvement of both lipids and proteins during inhibition by rotenone. The present study suggests that the lipid involvment may be more important in inhibition as evidenced by the solubility behaviour of 12a-hydroxypachyrrhizone (5) and 12a-hydroxydolineone (3). The former has a higher solubility in petroleum ether than the latter (using this principle 12a-hydroxydolineone could be fractionally

crystallized from admixture with 12a-hydroxypachyrrhizone in $CHCl_3$ solution by adding petroleum ether) and has relatively greater inhibitor potency both for inhibition of glutamate (TABLE III) and general respiration (TABLE I). At lower concentrations the low activity of 12a-hydroxydolineone (3) and 12a-hydroxypachyrrhizone(5) as compared with dolineone (2) and pachyrrhizone(4) may be attributed to a slight conformational change at B/C ring junction owing to introduction of an extra OH at 12a position. The conformational change is clear in N.M.R. studies which show a upfield shift of stereochemically specific C-1 proton signal (CROMBIE and LOWN 1962) by $\sim$ 0.25 ppm on introduction of 12a-OH group for a number of rotenoids (MANGAL NATH 1978). For instance in N.M.R. spectrum of dolineone, signal for C-1 proton appears at $\delta$ 6.73 but similar signal for 12a-hydroxydolineone is observed at $\delta$ 6.48. Once these 12a-hydroxyrotenoids start inhibiting to a significant extent, their detoxication rates are low (YAMAMOTO et al. 1971), they are very slowly metablised, in turn accumulate leading to more marked increase in inhibition as compared to pachyrrhizone (4) and dolineone (2); which seem to have better detoxication rates.

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