The Inhibition of Photoreactions of Chloroplasts by 2-Alkyl-4-hydroxyquinoline N-Oxides

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An antagonist of streptomycin action in several bacteria was shown to consist of a mixture of 2-alkyl-4-hydroxyquinoline N-oxides (Cornforth & James, 1956). The same compounds were demonstrated to be effective inhibitors of electron transport in cell-free preparations of heart muscle and some bacterial preparations (Lightbown & Jackson, 1956; Jackson & Lightbown, 1958). The site of the inhibitory action of 2-heptyl-4-hydroxyquinoline N-oxide in heart-muscle preparations was concluded to be between diaphorase and cytochrome c, and most probably between cytochrome b and cytochrome c (Jackson & Lightbown, 1958).

More recently, the same compound was observed (Smith & Baltscheffsky, 1959) to inhibit completely at 1 μm concentration light-induced phosphorylation in extracts of the photosynthetic bacterium Rhodospirillum rubrum. At the same concentration it had no effect on the respiratory activity of the extract. In addition, it produced changes in the light-dependent difference spectrum which were interpreted as an increased oxidation of cytochrome $c_2$ and a decreased oxidation of the $b$-type cytochromes. 2-Heptyl-4-hydroxyquinoline N-oxide has also been used as an accelerator of photophosphorylation (Vernon, 1959) and inhibitor of photoreduction (Vernon & Ash, 1959) catalysed by bacterial chromatophores. A communication describing its inhibition of photophosphorylation catalysed by spinach chloroplasts has recently appeared (Baltscheffsky, 1959).

In view of the potency of these compounds in specifically inhibiting the bacterial photophosphorylative system, it was thought of importance to determine their effect on the different photoreactions catalysed by higher plant chloroplasts. Two of the most potent of this group of inhibitors were selected (Lightbown & Jackson, 1956), and a quantitative study of their inhibitory action was undertaken. Experiments designed to test their specificity, site of action, manner of attachment and relation to other inhibitors of photoreactions in chloroplasts are described below.

METHODS

Preparation of chloroplasts. Chloroplasts and chloroplast fragments were prepared from greenhouse-grown Swiss-chard leaves as described by Avron (1960).

Measurement of reactions. The procedure followed and the measurement of the production of adenosine [32P]triphosphate (ATP) were as described by Avron (1960). The reduction of ferricyanide was determined from the decrease of $E$ at 420 mμ of a sample of the trichloroacetic acid-denatured supernatant, a molar absorption coefficient of $1.06 \times 10^3$ being used. The reduction of triphosphopyridine nucleotide (TPN) and trichloroindophenol was determined from the change of $E$ at 240 and 620 mμ respectively of the complete reaction mixture. All changes in $E$ were measured against a blank which contained the same reaction mixture, but to which no dye had been added.

All reactions were run at 17°, with air as the gas phase. The reaction cuvettes were placed within the aquarium described by Avron (1960), with an illumination of about 150,000 lux of white light.

Solutions of inhibitor. The inhibitor was dissolved in mm-NaOH solution and added as such. Control samples contained an equivalent amount of mm-NaOH. The concentration of the inhibitor in the stock solution was determined before each experiment by its absorption at 340 mμ (Corn-
The effects of several concentrations of 2-heptyl-4-hydroxyquinoline N-oxide on the reduction of ferricyanide and the associated phosphorylation (Avron, Krogmann & Jagendorf, 1958) are illustrated in Fig. 1. Both the phosphorylation and the reduction were inhibited to a similar extent. In general, the phosphorylation seemed to be slightly more sensitive to these inhibitors than the reduction. This was true with either ferricyanide or TPN as the electron acceptor.

Fig. 1 also demonstrates the fast action of these inhibitors. There was never any increase in inhibition observed as the reaction progressed, indicating that the interaction of the inhibitors with the active site in the chloroplasts was rapid and did not require a preincubation period. This is particularly noteworthy in view of the extremely low concentrations of these compounds required for inhibition.

The effect of increasing concentrations of 2-heptyl-4-hydroxyquinoline N-oxide was examined on the following types of photoreactions catalysed by higher-plant chloroplasts: (1) photophosphorylation dependent upon the presence of one of several cofactors (Jagendorf & Avron, 1958); (2) reduction of trichloroindophenol in the Hill reaction; (3) reduction of TPN or ferricyanide, and accompanying phosphorylations (Avron & Jagendorf, 1959). It is clear that these reactions fall into three groups (Fig. 2). The phenazine methosulphate-dependent photophosphorylation comprises the first, least-sensitive group. Concentrations up to 20 μM have little or no inhibitory effect in this reaction. Intermediate sensitivity was shown by most of the reactions tested, with 50% inhibition occurring around 10 μM. In this intermediate range fall the photophosphorylations dependent upon the presence of flavin mononucleotide and vitamin K₃ (Fig. 2). In addition, the photophosphorylation...
dependent upon indigo carmine, reduced trichloroindophenol (Krogmann & Vennesland, 1959; with our preparations this reaction did not show a strict oxygen-dependence) and the low phosphorylation present in the absence of any cofactor (Avron, 1960), fall in this intermediate category (not shown in the Figure). The Hill reaction, with trichloroindophenol as an electron acceptor, and the reduction and accompanying phosphorylation of TPN, could also be placed with the intermediate group.

The reaction most sensitive to 2-heptyl-4-hydroxyquinoline N-oxide was the reduction, and accompanying phosphorylation, of ferri cyanide. Here, 50 % inhibition was reached at a concentration of about 1 \( \mu \text{M} \). As shown in Fig. 1, the inhibition of the reduction of ferri cyanide and the accompanying phosphorylation were similar.

Fig. 3 illustrates the data obtained when the heptyl derivative was replaced by its more potent analogue 2-nonyl-4-hydroxyquinoline N-oxide. In all these reactions, as in its action in antagonizing dihydrostreptomycin activity (Lewitton & Jackson, 1966), the nonyl derivative was about five to ten times as effective as the heptyl compound. Here again, phenazine methosulphate-dependent phosphorylation was least sensitive, but small

![Graph](image)

Table 1. Dependence of inhibition of phosphorylation by 2-heptyl-4-hydroxyquinoline N-oxide upon chloroplast concentration

<table>
<thead>
<tr>
<th>Chloroplast concn. (mg. of chlorophyll/ml)</th>
<th>2-Heptyl-4-hydroxyquinoline N-oxide concn. (( \mu \text{M} ))</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-9</td>
<td>0-4</td>
<td>53</td>
</tr>
<tr>
<td>13-9</td>
<td>0-4</td>
<td>50</td>
</tr>
<tr>
<td>26-8</td>
<td>0-4</td>
<td>91</td>
</tr>
<tr>
<td>26-8</td>
<td>0-8</td>
<td>76</td>
</tr>
</tbody>
</table>

inhibitions could be observed above a concentration of 5 \( \mu \text{M} \). The same reactions fell into the intermediate-sensitivity group, as with the heptyl derivative, but 50 % inhibition was obtained at about 1 \( \mu \text{M} \) concentration. Also the photophosphorylation in the absence of any cofactor, and those dependent upon the presence of indigo carmine and reduced trichloroindophenol, were intermediate in sensitivity. The reduction of ferri cyanide and the accompanying phosphorylation were most sensitive; 50 % inhibition was reached at about 0-2 \( \mu \text{M} \)-2-nonyl-4-hydroxyquinoline N-oxide.

Variation in some of the early experiments on the action of these inhibitors was traced to the concentration of the chloroplasts in the experimental flasks (Table 1). Doubling the concentration of the chloroplasts lowered the inhibition of the photophosphorylation dependent upon ferri cyanide reduction from 47 % to 20 %. Further doubling of the chloroplast concentration decreased it to 9 %. It was therefore important to compare the extent of inhibitions of different reactions at similar chloroplast concentrations, whenever possible.

The effects of the heptyl- or nonyl-hydroxyquinoline N-oxides were also tested with chloroplast fragments (Avron, 1960) as well as whole chloroplasts. The reactions studied were the photophosphorylations dependent upon phenazine methosulphate, flavin mononucleotide, indigo carmine, reduced indophenol and no cofactor, and the reduction and accompanying phosphorylation of ferri cyanide. In no case was there any substantial difference between these two types of preparations.

Since ferri cyanide-dependent photoreactions were most severely inhibited, it was thought desirable to eliminate the possibility of a direct interaction between ferri cyanide and the inhibitors as a factor in their increased susceptibility. The effect of
2-heptyl-4-hydroxyquinoline N-oxide was tested therefore on the action of reduced triphosphopyridine nucleotide (TPNH) diaphorase (Avron & Jagendorf, 1956), isolated from the same leaves, with ferricyanide or trichloroindophenol acting as electron acceptors. No inhibition by 15 µM 2-heptyl-4-hydroxyquinoline N-oxide of TPNH diaphorase was observed with either electron acceptor.

The sensitivity of the different photoreactions to 2-heptyl-4-hydroxyquinoline N-oxide and 2-nonyl-4-hydroxyquinoline N-oxide was similar to that previously observed with another potent inhibitor, 3-(p-chlorophenyl)-1:1-dimethylurea (Jagendorf & Avron, 1959). This similarity is evident in the resistance exhibited by the phenazine methosulphate-mediated phosphorylation to both inhibitors, as compared with the other photoreactions studied. Since the site of action of 3-(p-chlorophenyl)-1:1-dimethylurea has been indicated by several different types of experiments to be on the oxygen-evolution path (Bishop, 1958; Jagendorf & Avron, 1959; Vernon & Zaugg, 1960), several experiments were designed to test further the identity in site of action of the two inhibitors.

It was previously shown (Avron & Jagendorf, 1959) that although phenazine methosulphate-dependent phosphorylation was insensitive to 3-(p-chlorophenyl)-1:1-dimethylurea when added alone, it was completely inhibited by the same concentration of 3-(p-chlorophenyl)-1:1-dimethylurea when added with small amounts of ferricyanide. In this respect, too, 2-heptyl-4-hydroxyquinoline N-oxide behaved similarly to 3-(p-chlorophenyl)-1:1-dimethylurea; the reaction was completely inhibited in the presence of ferricyanide and 2-heptyl-4-hydroxyquinoline N-oxide, but not in the presence of either compound alone (Fig. 4). If 3-(p-chlorophenyl)-1:1-dimethylurea and the quinoline N-oxides act in the identical site on the chloroplasts, it would be expected that their action would be additive. That is, when a concentration of 3-(p-chlorophenyl)-1:1-dimethylurea was added to one of 2-nonyl-4-hydroxyquinoline N-oxide, each of which caused the same inhibitory effect when added alone, it would be expected that the resulting inhibition would be equivalent to that produced by doubling the concentration of each inhibitor by itself. If, on the other hand, the two were acting at two different sites on the same chain, the decrease in activity caused by adding the two inhibitors together should not exceed that which was produced by each one by itself. Table 2 shows the results of the experiment described. The inhibition produced by the presence of both inhibitors together was equivalent to that produced by double the amount of each by itself, pointing to the identity of their site of attack.

The reversal, by washing, of the inhibitory effect of 3-(p-chlorophenyl)-1:1-dimethylurea has been previously described (Wessels & Van Der Veen, 1956; Spikes, 1956). The effect of the quinoline N-oxides was, similarly, easily reversed by washing of the chloroplast preparation; 80-90% of the activity was recovered after a single wash of chloroplasts treated with 0.5 µM 2-nonyl-4-hydroxyquinoline N-oxide.

![Fig. 4. Effect of ferricyanide on the inhibition of phenazine methosulphate-dependent phosphorylation by 2-heptyl-4-hydroxyquinoline N-oxide. Reaction mixture was as described under Fig. 1, except for the substitution of 33 µM phenazine methosulphate for K₃Fe(CN)₆. Chloroplasts contained 48 µg. of chlorophyll/ml. of reaction mixture. ○, Control; △, 3-2 µM 2-heptyl-4-hydroxyquinoline N-oxide; ▲, 0.33 mM K₃Fe(CN)₆; △, 3-2 µM 2-heptyl-4-hydroxyquinoline N-oxide and 0.33 mM K₃Fe(CN)₆.](image-url)
DISCUSSION

The inhibitory effect of the 2-alkyl-4-hydroxyquinoline N-oxides in the photoreactions catalysed by chloroplasts was quite specific. The inhibitors could not directly interact with the phosphorylation mechanism or the electron-transport steps closely associated with it. This was concluded since the most rapid photophosphorylation reaction known (i.e. in the presence of phenazine methosulphate) was by far the least sensitive. Their site of action must be directly involved therefore in the electron-transport steps necessary for the Hill reaction and the photophosphorylations requiring co-factors other than phenazine methosulphate, but only indirectly, if at all, in the photophosphorylation catalysed by phenazine methosulphate.

The similarity between the pattern of inhibitory action of 3-(p-chlorophenyl)-1:1-dimethyurea (Jagendorf & Avron, 1959) and the quinoline N-oxides (Figs. 2, 3), and the additivity of their action (Table 2), suggest that the site of action of the two is identical. It would seem therefore that the heptyl- and nonyl-hydroxyquinoline N-oxides inhibit a step in the path of oxygen evolution.

The reduction and accompanying phosphorylation of ferricyanide were most severely affected by the inhibitors studied. In this case, 2-nonyl-4-hydroxyquinoline N-oxide, which reduces the control rate by 50% at 0.2 μM, would be equal in its potency to the most active inhibitors of the reaction known (Wessels & Van der Veen, 1956). It may be worth emphasizing that at 0.2 μM the concentration of the inhibitor was several-fold lower than that of chlorophyll in the reaction mixture, even though the concentration of the chloroplasts used was very low and limiting the rate of the reaction. This supports the suggestion that only part of the chlorophyll within the chloroplast is active in catalysing the photoreaction studied.

The reason for the high sensitivity of the ferricyanide-dependent reactions, as compared with the others investigated, is not clear. However, the manner in which Figs. 2 and 3 were drawn tends to obliterate the fact that these reactions differ considerably in their rates. In terms of μmoles of ATP synthesized/mg. of chlorophyll/hr. the photophosphorylation dependent on phenazine methosulphate has a rate of 2000, on ferricyanide 200, and on TPN 20. In this light, the resistance of the photophosphorylation dependent on phenazine methosulphate becomes even more significant, and the difference between those dependent on ferricyanide and TPN may only be a reflexion of the difference in the rates of the respective reactions.

SUMMARY

1. 2-Heptyl-4-hydroxyquinoline N-oxide and 2-nonyl-4-hydroxyquinoline N-oxide were found to be potent inhibitors of some of the photoreactions catalysed by Swiss-chard chloroplasts. Their quantitative effects are reported on the Hill reaction, cyclic photophosphorylation in the presence of different catalysts and open photophosphorylation in the presence of ferricyanide or triphosphopyridine nucleotide.

2. By far the most resistant reaction was the cyclic photophosphorylation dependent upon the presence of phenazine methosulphate. The most sensitive were the reduction and associated phosphorylation of ferricyanide. Inhibition of the latter by 50% was observed with 0.2 μM concentrations of the nonyl derivative.

3. By analogy with the inhibition caused by 3-(p-chlorophenyl)-1:1-dimethyurea, it is suggested that the quinoline N-oxides react with a site on the oxygen-evolution pathway.

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REFERENCES