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# Studies on Effect of Certain Quinones

## I. ELECTRON TRANSPORT, PHOTOPHOSPHORYLATION, AND $CO_2$ FIXATION IN ISOLATED CHLOROPLASTS[1]

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### ABSTRACT

**The effect of quinone herbicides and fungicides on photosynthetic reactions in isolated spinach (*Spinacia oleracea*) chloroplasts was investigated. 2,3-Dichloro-1,4-naphthoquinone (dichlone), 2-amino-3-chloro-1,4-naphthoquinone (06K-quinone), and 2,3,5,6-tetrachloro-1,4-benzoquinone (chloranil) inhibited ferricyanide reduction as well as ATP formation. Benzoquinone had little or no effect on these reactions. The two reactions showed a differential sensitivity to these inhibitors. Dichlone was a strong inhibitor of both photosystems I and II; photosystem I was more sensitive to 06K-quinone than was photosystem II, whereas the reverse was true of chloranil. Chloranil and 06K-quinone inhibited ferricyanide reduction and the coupled photophosphorylation to the same extent, whereas dichlone affected photophosphorylation to a greater extent than the ferricyanide reduction.**

**$CO_2$ fixation was inhibited by all the quinones to varying degrees. In chloroplasts treated with 06K-quinone or benzoquinone, $CO_2$ fixation was inhibited to a greater extent than the photoreduction of ferricyanide or ATP formation, indicating the possibility that the two quinones may also inhibit certain reactions in the carbon reduction cycle. The effect of dichlone and chloranil, but not of 06K-quinone, was overcome by the addition of reduced glutathione. The quinones caused an increase in the proportion of $^{14}C$ incorporated into 3-phosphoglyceric acid and a reduction in the amount of glycolic acid.**

A number of quinonoid compounds have been found to have fungicidal, algicidal, and herbicidal effects. The fungicidal activity of substituted benzoquinones and naphthoquinones was studied and has been found to be related to the inhibition of enzymes and coenzymes with free sulfhydryl and amino groups (11, 14). Very little information is available on the mode of action of quinone algicides or herbicides in algae and higher plants. Zweig *et al.* (16, 17) reported that certain quinones, herbicides, and algicides inhibited oxygen evolution and $CO_2$ fixation in *Chlorella*, suggesting that these chemicals interfere with photosynthesis. In order to elucidate which site(s) in the photosynthetic process is affected by the quinones, the effect of these inhibitors on photosynthetic reactions such as electron transport, photophosphorylation, and $CO_2$ fixation was investigated in isolated chloroplasts. The quinones tested in this study included 2,3-dichloro-1,4-naphthoquinone, 2-amino-3-chloro-1,4-naphthoquinone, 2,3,5,6-tetrachloro-1,4-benzoquinone, and 1,4-benzoquinone.

### MATERIALS AND METHODS

For electron transport and photophosphorylation studies, the chloroplasts were prepared from spinach (*Spinacia oleracea*) leaves as described by Avron (2). The chloroplasts were washed once with the sucrose-tris-NaCl homogenizing medium without ascorbate. Chlorophyll was determined by the method of Arnon (1).

**Measurement of Ferricyanide Reduction and Photophosphorylation.** The effect of quinones on the photoreduction of ferricyanide was measured under both nonphosphorylating (basal) and phosphorylating conditions. The procedure followed for the measurement of photophosphorylation was as described by Avron (2). The standard reaction mixture contained, in $\mu$moles: tris, pH 7.8, 45; NaCl, 60; $MgCl_2$, 12; sodium-potassium phosphate, pH 7.8, 12; ADP, 12; adequate $^{32}P$ (about $10^6$ cpm); PMS[3], 0.09 (cyclic photophosphorylation); or potassium ferricyanide, 0.5 (noncyclic); chloroplast suspension containing between 25 and 50 $\mu$g of chlorophyll, quinone solution, and water to a total volume of 3.0 ml. In the measurements of basal electron transport, phosphorylating reagents (ADP, $Mg^{2+}$, and $P_i$) were omitted from the reaction mixture. The tubes containing the reaction mixtures were placed in a water bath maintained at 15 C, and the reaction was started by turning the light on. The light intensity at the level of the tube was approximately 16,000 ft-c. After 2 min of illumination, the light was turned off, and each chloroplast suspension was inactivated quickly by the addition of 0.3 ml of 30% (w/v) trichloroacetic acid. The samples were centrifuged, and aliquots of supernatant were analyzed for $^{32}P$ (ATP), as described by Avron (2). Ferricyanide reduction was determined by measuring the absorption at 420 nm in centrifuged aliquots of the reaction mixture. P/2e ratio was calculated from the values of ferricyanide reduction and ATP formation.

**$CO_2$ Fixation.** For $CO_2$ fixation studies, chloroplasts were isolated from 4- to 6-week-old spinach grown in the field. The leaves were picked immediately prior to their use in experi-

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[3] Abbreviations: chloranil: 2,3,5,6-tetrachloro-1,4-benzoquinone; dichlone: 2,3-dichloro-1,4-naphthoquinone; 06K-quinone: 2-amino-3-chloro-1,4-naphthoquinone; PMS: phenazine methosulfate.

ments, and the chloroplasts were isolated according to the method described by Jensen and Bassham (10). $^{14}CO_2$ fixation was carried out in glass centrifuge tubes. The reaction mixture contained 0.33 M sorbitol, 2 mM $NaNO_3$; 2 mM $K_2EDTA$; 2 mM sodium isoascorbate; 1 mM $MnCl_2$; 1 mM $MgCl_2$, 0.5 mM $K_2HPO_4$; 50 mM HEPES, adjusted with NaOH to pH 7.6; 5 mM $Na_4P_2O_7 \cdot H_2O$, chloroplast suspension containing 100 to 150 $\mu$g of chlorophyll in a total volume of 1 ml. Quinone solution in ethanol was added to the tube so that the final concentration of ethanol in the reaction mixture was 1%. The reaction mixture in the control tubes contained a similar amount of ethanol. It was shown that this concentration of ethanol had no effect on any of the reactions described below. The tubes were placed in the water bath maintained at 20 C and preilluminated for 3 min. The light intensity at the surface of the reaction mixture was about 2500 ft-c. $NaH^{14}CO_3$ solution was then added to each tube to make the solution 0.4 mM in $HCO_3^-$. After 10 min of photosynthesis, the reaction was stopped by addition of 4 ml of absolute ethanol, the extract was centrifuged, and the supernatant was removed. The pellet was re-extracted with 50% ethanol. An aliquot of the combined extract was acidified with HCl and counted for radioactivity in a liquid scintillation counter.

For separation of the photosynthetic products, the chloroplast extract was concentrated and an aliquot containing at least 30,000 dpm was chromatographed two dimensionally as described by Pedersen *et al.* (12). The aliquot volume varied from 20 to 100 $\mu$l depending on total $^{14}C$ fixed. Immediately after developing in the second solvent, the area of the paper where glycolic acid migrates was sprayed with $NaHCO_3$. The chromatograms were exposed to x-ray film, and autoradiographs were developed. The radioactivity of each spot was determined by cutting it out, placing it in a vial containing the scintillation fluid, and counting in the scintillation counter.

Table I. *Effect of Quinones on Ferricyanide Reduction and Ferricyanide and PMS Photophosphorylation*

Chloroplasts were prepared by the method of Avron (2). Ferricyanide reduction and photophosphorylation were carried out in quinone-treated chloroplasts, as described in the text. After 2 min of illumination, the reaction was stopped by the addition of trichloroacetic acid, and the ferricyanide reduction and the amount of $^{32}P$ incorporated was determined. Ferricyanide reduction was 335 $\mu$moles per mg chl per hr and 445 in the presence of phosphorylating reagents. Ferricyanide-mediated photophosphorylation was 162 $\mu$moles of ATP formed per mg chlorophyll per hour. PMS phosphorylation was 302 $\mu$moles of ATP formed per mg chl per hr. The P/2e ratio was 0.73.

| Quinone Concn | | Ferricyanide | | | P/2e[2] | PMS-dependent ATP Formation |
|---|---|---|---|---|---|---|
| | | Reduction[1] | Reduction | ATP formation | | |
| | $\mu M$ | | % control | | | % control |
| Dichlone | 30 | 50 | 36 | 11 | 0.32 | 26 |
| Dichlone | 3 | | 77 | 36 | 0.48 | 76 |
| Chloranil | 30 | 26 | 24 | 15 | 0.60 | 57 |
| Chloranil | 3 | | 92 | 92 | 1.00 | 93 |
| 06K-Quinone | 30 | 66 | 64 | 71 | 1.10 | 22 |
| 06K-Quinone | 3 | | 97 | 91 | 0.94 | 56 |
| Benzoquinone | 30 | | 100 | 100 | 1.00 | 76 |
| Benzoquinone | 3 | | 100 | 100 | 1.00 | 100 |

[1] No phosphorylating reagents.

[2] Corrected P/2e ratio, setting the control at a theoretical value of 1.0.

## RESULTS AND DISCUSSION

**Effect of Quinones on Ferricyanide Reduction and Associated Photophosphorylation.** Table I shows the effects of different quinones on ferricyanide reduction and the accompanying photophosphorylation (photosystem II). Both ferricyanide reduction and photophosphorylation were inhibited by the quinones; dichlone and chloranil at 30 $\mu$M severely inhibited the two reactions, whereas 06K-quinone was a relatively weak inhibitor. Chloranil and 06K-quinone inhibited ferricyanide reduction and the coupled photophosphorylation in an approximately parallel fashion. However, in chloroplasts treated with dichlone, photophosphorylation was more severely inhibited than the ferricyanide reduction, resulting in a low P/2e ratio. Inhibition of both electron transport and the accompanying photophosphorylation may result either from effect on the electron transport chain *per se* or on a site in the energy transfer reactions leading to ATP formation which is tightly coupled to electron transport. It was observed that the quinones inhibited ferricyanide reduction both in the absence and presence of ADP, indicating that the inhibition of ATP formation is the result of electron flow inhibition. It is likely that quinones inhibited electron transport by competing with ferricyanide for electrons since most of the quinones included in the study have a more positive redox potential than ferricyanide (17). A greater inhibition of photophosphorylation than that of ferricyanide reduction in the dichlone-treated chloroplasts seems to indicate that this quinone also acts on a site in energy transfer reactions in addition to inhibiting the electron transport system.

The fact that these quinones could be photoreduced by illuminated chloroplasts, as demonstrated by Cho *et al.* (8), indicates that these inhibitors do not block the primary electron flow from water. On the basis of the available data, we suggest that the inhibition of electron transport to ferricyanide is not the result of inhibition of electron flow from water, as in the case of DCMU, but rather is due to a block in the flow of electrons at other sites of the transport chain, presumably after the water splitting site, or to a divergence of electron flow to electron acceptors other than ferricyanide.

**Effects of Quinones on Cyclic Photophosphorylation.** Table I shows that photosphosphorylation catalyzed by PMS (photosystem I) was inhibited by dichlone, chloranil, and 06K-quinone at 30 $\mu$M. In contrast to its moderate effect on noncyclic photophosphorylation, 30 $\mu$M 06K-quinone strongly inhibited PMS-dependent photophosphorylation. The different quinones at a concentration of 3 $\mu$M inhibited the cyclic photophosphorylation in the following order of effectiveness: 06K-quinone > dichlone > chloranil > benzoquinone. At the higher concentration, both 06K-quinone and dichlone were almost equally effective in inhibiting PMS-dependent photophosphorylation. Zweig *et al.* (17) have demonstrated that 06K-quinone inhibits cyclic photophosphorylation by competing with PMS for electrons in the transport chain. On the basis of this evidence, it is assumed that other quinones tested in this study may inhibit cyclic photophosphorylation like 06K-quinone.

A comparison of the relative inhibition of ferricyanide and PMS-dependent phosphorylations by different quinones shows that these chemicals affect the two systems to different extents. Dichlone and chloranil inhibited the noncyclic photophosphorylation more strongly than they did the cyclic photophosphorylation, whereas the reverse was true of 06K-quinone. A possible explanation for this differential inhibition of two phosphorylating systems is the existence of two different sites of photophosphorylation as suggested by Avron and Neumann (3) and Avron and Shavit (4) and their differential sensitivity to the quinones. If the hypothesis concerning the existence of

two phosphorylation sites is true, then these quinones appear to act at more than one site in the light reactions in isolated chloroplasts.

Benzoquinone showed no effect on ferricyanide reduction and the associated photophosphorylation, but slightly affected PMS-dependent photophosphorylation.

**Effect of Quinones on $^{14}CO_2$ Fixation.** Table II shows the effect of different concentrations of quinones on $^{14}CO_2$ fixation by spinach chloroplasts. All the quinones at 30 $\mu$M inhibited $CO_2$ fixation. 06K-quinone was observed to be the most inhibitory, followed in order by dichlone, benzoquinone, and chloronil.

Since photosynthetic $CO_2$ fixation is governed, among other factors, by the generation of ATP and TPNH, we will try to establish if the inhibition of $CO_2$ fixation in chloroplasts treated with quinones can be attributed to the effect of these inhibitors on the supply of ATP and TPNH. It should, however, be borne in mind that the effects of each inhibitor on the various reactions are not directly comparable because different procedures were used to isolate chloroplasts for studying different reactions. For electron transport and photophosphorylation experiments, chloroplasts were prepared according to the method of Avron (2), whereas for $CO_2$ fixation, Jensen and Bassham's procedure (10) was used to isolate chloroplasts. There were also inevitable differences in the techniques used to study each process.

Dichlone and chloranil were strong inhibitors of the Hill reaction and of photophosphorylation; the reduction in $CO_2$ fixation by these two chemicals can be explained by the fact that they inhibited the light reactions which supply ATP and reducing power required for $CO_2$ fixation. On the other hand, the inhibition of $CO_2$ fixation in the presence of 06K-quinone and benzoquinone cannot be entirely attributed to a shortage of ATP and reducing power since these chemicals inhibited $CO_2$ fixation more severely than electron transport or photophosphorylation. At a concentration of 3 $\mu$M, 06K-quinone showed little or no effect on electron transport or non-cyclic photophosphorylation, moderately affected cyclic photophosphorylation, but almost completely blocked $CO_2$ fixation. In chloroplasts treated with benzoquinone, little or no effect was observed on electron transport or photophosphorylation, but there was a strong inhibition of $CO_2$ fixation. On the basis of these findings, it is suggested that benzoquinone and 06K-quinone may also inhibit certain reactions in the carbon reduction cycle.

**Effect of Quinones on Products of $^{14}CO_2$ Fixation in Chloroplasts.** The effect of different quinones on the incorporation of $^{14}CO_2$ into the products of photosynthesis is shown in Table III. The products of $^{14}CO_2$ fixation in chloroplasts consisted mainly of the intermediates of the carbon reduction cycle and glycolic acid, with a large proportion of the $^{14}CO_2$ incorporated in 3-phosphoglyceric acid and glycolic acid. These results are in agreement with those of Ellyard and Gibbs (9) who reported that high $O_2$ and low bicarbonate concentration resulted in an increase in the percentage of $^{14}C$ incorporated into glycolic acid. The quinones not only inhibited the fixation of $CO_2$ by the chloroplasts, but also had a pronounced effect on the distribution of $^{14}C$ among the products of fixation. Most noticeable was an accumulation of 3-phosphoglyceric acid accompanied by a severe reduction in the amount of $^{14}C$-glycolic acid in the chloroplasts treated with various quinones. The proportion of $^{14}C$ in sugar mono- and diphosphates also showed an increase in the treated chloroplasts.

The exact mechanism by which quinones alter the flow of carbon into the products of fixation is not known. Studies are in progress to determine the site where inhibition of glycolic acid synthesis occurs. The biosynthetic pathway leading to the formation of glycolic acid is not yet fully established; it is generally assumed that glycolic acid is derived from the hexose phosphate of the carbon reduction cycle (6, 13). If this is true, then a rise in the level of 3-phosphoglyceric acid and sugar mono- and diphosphates, accompanied by a sharp decline in the level of glycolic acid, seems to suggest that the site of inhibition of glycolic acid by the quinones lies between hexose

Table II. *Effect of Quinones on $^{14}CO_2$ Fixation by Chloroplasts*

Chloroplasts were prepared by the method of Jensen and Bassham (10). The chloroplast suspension was incubated with desired concentrations of quinones for 3 min. $NaH^{14}CO_3$ was then introduced in the reaction mixture, and $^{14}CO_2$ fixation was carried out as described in the text. After 10 min of photosynthesis, the reaction was stopped by addition of absolute ethanol. Control rate for $CO_2$ fixation was 10 $\mu$moles per mg chl per hr.

| Quinone Concn | Dichlone | 06K-Quinone | Chloranil | Benzoquinone |
|---|---|---|---|---|
| $\mu$M | % control | | | |
| 30 | 1.0 | 1.0 | 48.0 | 8.4 |
| 3 | 52.1 | 5.3 | 86.6 | 90.2 |

Table III. *Effect of Quinones on the Relative Distribution of $^{14}C$-Products in Chloroplasts*

Chloroplast isolation and experimental conditions were as outlined in Table I. After 10 min of photosynthesis, the reaction was stopped and the products were separated by descending paper chromatography. The data represent the percentage of radioactivity detected on the paper chromatogram.

| Treatment | Total dpm | $^{14}C$ Incorporated Into | | | | |
|---|---|---|---|---|---|---|
| | | 3-Phosphoglyceric acid | Triose-P | Glycolic acid | Sugar monophosphates | Sugar diphosphates |
| | | % | | | | |
| Control | 76200 | 44.4 | 4.3 | 38.0 | 2.1 | 11.0 |
| Dichlone, 3 $\mu$M | 45660 | 61.6 | 8.2 | 8.0 | 5.4 | 15.6 |
| 06K-Quinone, 3 $\mu$M | 31040 | 64.2 | 7.0 | 0.8 | 8.1 | 19.1 |
| Benzoquinone, 30 $\mu$M | 37810 | 62.8 | 8.8 | 2.3 | 5.7 | 18.8 |

Table IV. *Effect of Quinones on $^{14}CO_2$ Fixation by Chloroplasts in the Absence or Presence of Glutathione*

Chloroplast isolation and experimental conditions were as outlined in Table I with the exception that the chloroplasts were incubated with 1 mM GSH for 5 min before the addition of quinone and $NaH^{14}CO_3$. Control rate for $CO_2$ fixation was 10 $\mu$moles per mg chl per hr.

| Compound Concn | Percentage of Control |
|---|---|
| Dichlone, 3 $\mu$M | 50.2 |
| Dichlone, 3 $\mu$M + GSH | 101.4 |
| Chloranil, 30 $\mu$M | 60.8 |
| Chloranil, 30 $\mu$M + GSH | 99.1 |
| 06K-Quinone, 1.5 $\mu$M | 15.7 |
| 06K-Quinone, 1.5 $\mu$M + GSH | 15.1 |
| Benzoquinone, 30 $\mu$M | 16.6 |
| Benzoquinone, 30 $\mu$M + GSH | 48.3 |
| GSH | 99.2 |

phosphate and glycolic acid. The pattern of distribution of $^{14}C$-products indicates that the quinones interfere with certain reactions in the carbon reduction cycle.

**Reversal of the Quinone Effect by Glutathione.** Quinones are known to react readily with thiols and certain amines, and it has been suggested that reactions with the free sulfhydryl and amino groups of enzymes may account for the phytotoxicity of quinones (11). There is evidence which shows that sulfhydryl enzymes are present in the chloroplasts (4, 15). If these enzymes are inactivated by quinones, certain thiols like GSH or cysteine should be able to prevent or reverse the inhibitory effect of the quinone. It was, therefore, of interest to determine if GSH can provide a protection against the quinones. The chloroplasts were incubated with GSH for 3 min before the addition of quinones and $NaH^{14}CO_3$. Table IV shows the effect of quinones on $^{14}CO_2$ fixation by the chloroplasts in the presence of GSH. GSH completely prevented the inhibition of $CO_2$ fixation resulting from the addition of dichlone and chloranil. These results seem to indicate that the toxicity of dichlone and chloranil results from their inactivation of one or more sulfhydryl compounds in the chain of reactions concerned with electron transport, photophosphorylation, and $CO_2$ fixation. In contrast to its complete protection against dichlone or chloranil, GSH was ineffective against 06K-quinone while providing only a partial protection against benzoquinone. This observation suggests the possibility that benzoquinone and 06K-quinone may inhibit photosynthetic reactions by reacting with functional groups other than the sulfhydryl ones of the various enzymes and co-enzymes in the chloroplasts. These results indicate that 06K-quinone and benzoquinone act at sites different from those at which dichlone or chloranil act.

A comparison of the effects of the different quinones on various photosynthetic reactions in chloroplasts indicates similarities, as well as differences, in their mode of action. All of them inhibit the $CO_2$ fixation in isolated chloroplasts, but the mechanism by which they bring about this inhibition does not appear to be similar.

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