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EFFECT OF QUINONES ON $NADPH_2$ OXIDATION AND PHOTOSYNTHETIC CO_2 ASSIMILATION IN *CHLORELLA PYRENOIDOSA**

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*Chlorella Pyrenoidosa*에 있어서 $NADPH_2$의 酸化와 光合成에 依한 CO_2 同化作用에 對한 Quinone의 影響

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要 約

1. $NADPH_2$酸化酵素存在下에 있어서 Quinone에 依한 $NADPH_2$의 酸化를 試驗管內에서 調査하였다. 그 結果를 3×10^{-5} M.의 各種 Quinone 類로 $^{14}CO_2$ 固定 10分前及固定試驗中 處理한 *Chlorella*의 $^{14}CO_2$ 固定率과 比較하였다.

2. 各種 Quinone 類의 $NADPH_2$ 酸化速度와 $^{14}CO_2$ 固定率사이에는 密接한 關係가 있음이 밝혀졌다. $NADPH_2$ 酸化와 $^{14}CO_2$ 固定에 對한 Quinone의 影響은 그 세기가 다음 順序와 같다.

Dichlone>06-K>NQ>BQ

3. *Chlorella*에 對한 Quinone의 毒性은 NADPH를 植物體에서 없앰으로서 $^{14}CO_2$ 固定을 妨害하여 細胞를 結果的으로 죽이는 것이라고 생각이된다.

4. *Chlorella*의 아미노酸 生合成에 對한 Quinone類의 影響은 全般的으로 이를 抑制하는 것이라 하겠다. 이것은 特히 Dichlone에 있어서 顯著하다. 이 現象은 植物에 $NADPH_2$가 없어지고 $^{14}CO_2$ 固定이 阻害되기 때문일 것이다. 蔗糖의 生合成은 影響을 받지않거나 오히려 刺戟을 받는데 그 理由는 不分明하다.

Introduction

It has been well known that quinones inhibit the growth of microorganisms due to their inhibitory action on sulfhydryl enzymes and redox-enzyme systems. In our previous research[1] it was shown that quinones also inhibited photosynthetic activities of *Chlorella pyrenoidosa* and eventually brought

* Abbreviations used:
BQ=1,4-benzoquinone
NQ=1,4-naphthoquinone
Diquat=6,7-dihydrodipyrido [1,2 a:a', 1'-c]
=pyrazidinium salt
06K-quinone=2-amino-3 chloro- 1, 4-naphthoquinone
Dichlone=2,3-dichloro-1, 4-benzoquinone
Chlorani=2,3,5,6 -tetrachloro-1, 4-benzoquinone
DCMU=3(3, 4-dichlorophenyl) 1, 1-dimethylurea
Atrazine=2-chloro- 4-ethylamino-6-isopropylaminos-triazine

death to this algae. Recently Zweig, *et al.*[2] and Black, *et al.*[3] have advanced the theory that the cause of phytotoxicity of diquat and some quinones might be due to their deprival of NADPH by the catalytic oxidation of reduced pyridine nucleotides in the quinones.

In the experiments reported here we have tested the oxidizability of $NADPH_2$ by various quinones in the presence of $NADPH_2$ diaphorase and air in a search for possible correlation between phytotoxicity of quinones and the oxidation of $NADPH_2$ catalyzed by quinones. These results are reported here together with the pattern of distribution of ^{14}C-amino acids, following $^{14}CO_2$-fixation by *Chlorella pyrenoidosa*.

Experimental

Enzymic Oxidation of $NADPH_2$ by quinones in the presence of $NADPH_2$ diaphorase.

The reaction mixture contains 0.3 μmols NADP H_2, 0.1 ml $0.9 \cdot 10^{-3}$ M quinone, 0.04 ml $NADPH_2$-diaphorase solution which contains 0.5 unit of enzyme activity per 0.68 mg protein; 0.3 ml 0.5 M Tris buffer of pH=7.5 and water to make total volume of 3 mls in silica cuvet, 1 cm-diam. The absorbance at 340 mμ for $NADPH_2$ was measured before and after the addition of quinones. The reaction mixture was gently shaken 3 times so that air would dissolve into the solution.

$^{14}CO_2$ fixation by *Chlorella pyrenoidosa*.

Chlorella pyrenoidosa suspension of 26 days old cells, grown according to the method described previously[1], was centrifuged at 500 × g and resuspended in distilled water to make the chlorophyll concentration 50 mg/l.

Into 5 mls *Chlorella* suspension in 150 ml Erlenmeyer flask, about 40 μc of $NaH^{14}CO_3$ with a specific activity of 40 μc/0.6 mg was added, and the flask was agitated on a rotary shaker under fluorescent light with an intensity of 3,000 lux. After 10 minutes illumination, 1 ml aliquot of the suspension was taken and put into 4 mls boiling ethanol in a 15 ml centrifuge tube. After 10 minutes extraction by boiling 80% EtOH, the solution was centrifuged at 500 × g, and the clear supernatant was evaporated under N_2 to a final volume of 0.5 ml for paper chromatographic separation of photosynthetates.

To test the effect of quinones on amino acid biosynthesis, $^{14}CO_2$-fixation experiments were performed in the presence of each quinone, by adding to 5 mls *Chlordlla* suspension 0.1 ml solution of $1.50 \cdot 10^{-3}$ M of each quinone. The cells were illuminated for 10 minutes in the shaker with gentle circular motion prior to the addition of radioactive sodium bicarbonate for $^{14}CO_2$-fixation.

Paper chromatography and autoradiography.

40 μl. aliquots of the 0.5 ml concentrated *Chlorella* extracts were spotted on 20 · 20 cm Whatman No. 1 filter paper and developed two dimensionally using Calvin and Bassham's solvents[4]. The developed paper chromatograms were exposed to Kodak-Blue Brand Medical X-ray film for one week.

Detrmination of radioactivity.

The radioactivity of total $^{14}CO_2$ fixed was determined as follows. Twenty μl of *Chlorella* suspension was spotted in a 1 cm-diameter filter paper disc and air-dried. Two or three drops of 10% acetic acid was applied on this spot, thus eliminating residual $^{14}CO_2$. After air-drying, the spot was counted by liquid scintillation spectrometry. The activity of $^{14}CO_2$-compounds distributed among various amino acids was counted using standard amino acid spots on paper chromatograms as guides.

Chemicals

$NADPH_2$ was purchased from Sigma Chemical Co., $NADPH_2$-diaphorase(*Cl. kluyveri*) was purchased from Worthington Biochemicals. 2-Methyl-1, 4-naphthoquinone was purchased from National Biochemical Corporation; 2, 3-dichloro-1, 4-naphthoquinone, 2-chloro-3-amino-1, 4-naphthoquinone, tetrachloro-*p*-benzoquinone were kindly supplied by Naugatuck Chemical Company. Diquat was kindly supplied by California Chemical Company. Benzoquinone and naphthoquinone were purified in our laboratory according to the usual method by sublimation and recrystallization from ethanol.

Results

Enzymic Oxidation of $NADPH_2$ by quinones.

The result of enzymic oxidation of $NADPH_2$ by quinones in the presence of $NADPH_2$-diaphorase

under aerobic conditions are summarized in Table 1. As the results show in Table 1, the catalytic

TABLE I. RATE OF ENZYMIC OXIDATION OF $NADPH_2$ BY QUINONES

Compound	$\triangle$ OD_{340} per minute	Rate of $NADPH_2$ disappearance (μmols/min.)
Dichlone	>>0.48*	>>2.67
06 K-quinone	0.420	2.33
Menadione	0.053	0.29
1, 4-Naphthoquinone	0.050	0.28
1, 4-Benzoquinone	0.045	0.25
Diquat	0.013	0.06

* Rapidly, cannot be determined accurately

action of quinones and diquat for enzymic oxidation of $NADPH_2$ are in the following sequence:

Dichlone>06K-quinone>menadione>NQ>BQ> diquat.

Total $^{14}CO_2$-fixation by *Chlorella*.

The amounts of total $^{14}CO_2$ fixed by *Chlorella* treated with various quinones are shown in Table 2. The order of inhibition by quinones is as follows:

TABLE II. EFFECT OF QUINONES, DIQUAT, AND DCMU ON $^{14}CO_2$-FIXATION BY *CHLORELLA*

Compound	Total $^{14}CO_2$ fixed. Radioactivity. c/m, for 20 ul suspension cpm	% of Control
Control	20, 273	100.0
Dichlone	1, 056	5.2
06-K quinone	12, 285	60.5
NQ	17, 149	84.5
BQ	17, 377	85.7
Diquat	14, 759	72.8
DCMU	2, 083	10.3

Dichlone>06 K-quinone>NQ>BQ

It seems that the degree of inhibition of $^{14}CO_2$-fixation is in the same sequence as the rate of $NADPH_2$ oxidation by quinones. Diquat and DCMU are included in this series and will be discussed below.

Effect on amino acid and sucrose synthesis by quinones in *Chlorella*.

The biosynthesis of amino acids and sucrose as affected by quinones is shown in Table 3. Gener-

TABLE III. EFFECTS OF QUINONES ON THE BIOSYNTHESIS OF AMINO ACIDS AND SUCROSE IN *CHLORELLA PYRENOIDOSA*

	Control	Dichlone	06-K	NQ	BQ	Chloranil	Diquat	DCMU
	Counts per minute (from 20 μl of original extract)							
Alanine	4, 993	484	2, 967	5, 190	4, 382	5, 803	3, 668	239
Glycine	1, 242	—	785	1, 375	1, 192	698	735	—
Serine	6, 081	137	3, 720	3, 747	4, 597	2, 027	3, 394	—
Sucrose	7, 064	602	10, 045	10, 019	10, 393	8, 489	4, 272	—
Glutamic Acid	1, 620	70	751	1, 126	636	719	1, 806	581
Aspartic Acid	3, 609	85	1, 560	1, 807	2, 293	193	4, 330	2, 017

ally, amino acids are depressed and sucrose synthesis is sometimes stimulated. However, it seems that dichlone is the most potent inhibitor of amino acid and sucrose synthesis.

Discussion

It has been postulated that quinones inhibit metabolic processes by combining with sulfhydryl enzymes or disturbing the redox enzyme systems[5]. In the latter case quinones might act as bridges or shunts in the electron transport of redox systems. If quinones acted as bridges of the electron transport system, they would not inhibit the metabolic processes. However, if quinones act as shunts in electron transport, they would divert the electrons from their normal path disturbing the redox system and inhibiting metabolism[5].

The results in Table 1 could best be explained by the second hypothesis of the above theories on quinone inhibition. In the primary action of phot-

osynthesis, quinones like plastoquinone and Vit K participate in the electron transport system[6]. It is possible that some quinone may fit structurally into the electron transport pathway while others do not. This may explain the reason for some quinones to be inhibitors of metabolism while others have no effect.

Menadione and naphthoquinone showed the same capability for $NADPH_2$ oxidation as seen in Table 1, but menadione shows no inhibitory action toward *Chlorella*[1] while naphthoquinone shows a severely inhibitory effect. This may be explained on the theory that menadione fits structurally into the photosynthetic electron transport system, thus facilitating electron flow without impedance. Naphthoquinone on the other hand does not seem to fit into the pathway serving as electron by-pass and shunting away electrons from the normal path, thus acting as an inhibitor.

Under aerobic conditions, NQ, 06 K-quinone, dichlone react with $NADPH_2$ to form NADP, and the reduced quinones are re-oxidized to their original state thus acting as catalysts. The plant system, in the presence of such quinones, may be deprived of $NADPH_2$ which is an essential component for CO_2-fixation by the Calvin cycle. The more readily NA DPH_2 is oxidized in the presence of a particular quinone, the smaller is the amount of available NA DPH_2: consequently less $^{14}CO_2$ will be fixed. The close correlation between the oxidizability of NAD PH_2 by quinones and their effect on $^{14}CO_2$-fixation in *Chlorella*, as shown in Table 2, could be explained by this line of reasoning.

The results of Table 3 showing the effect of quinones on amino acid and sucrose biosynthesis, may reflect the effect of $NADPH_2$-deprival and inhibition of $^{14}CO_2$ fixation by quinones in *Chlorella*. The results in Table 3 show a general depression of amino acid biosyn-thesis, which may occur by transamination from glutamic and aspartic acids and corresponding α-keto acids. Thus $NADPH_2$ is essentially needed for the synthesis of most amino acids. The general depression of amino acid biosynthesis by the presence of quinones could be explained again by the oxidation of $NADPH_2$ by quinones.

The stimulating effect by some quinones in the biosynthesis of sucrose cannot be explained at the present time and is the subject of future investigations.

Zweig *et al.*[7], Black and Myers[4] and Arriaga-Diaz[8] reported that diquat inhibits the photosynthesis of *Chlorella* by the same mechanism as described here for quinones. According to the experimental results obtained here, the $NADPH_2$ oxidizing ability of diquat is rather low. Also $^{14}CO_2$-fixation inhibition rate by diquat is rather low. It is possible, therefore, that the phytotoxicity of diquat in addition to the reasons discussed above, may be due to toxic free-radicals[9].

In the amino acids biosynthesis experiment we observed the depression of synthesis by chloranil, which was not tested for its $NADPH_2$-oxidizability. Though chloranil may manifest its phytotoxicity by depriving the plant of $NADPH_2$, the mechanism is by the non-enzymic oxidation of $NADPH_2$[10].

The explanation for the inhibition of $^{14}CO_2$-fixation by DCMU but the lack of a catalytic effect on the oxidation of $NADPH_2$ is that the site of action of DCMU lies directly at the oxygen-evolving step from water. This is similar to the action of atrazine on $^{14}CO_2$-fixation by excised bean leaves[11].

Summary

1. The oxidizability of $NADPH_2$ by quinones in the presence of $NADPH_2$-diaphorase was tested under aerobic conditions. Also the $^{14}CO_2$-fixation rates were compared when *Chlorella* suspensions were pretreated with $3 \cdot 10^{-5}$ M cocentration of variou quinones for 10 minutes prior and during the $^{14}CO_2$-fixation period.

2. A close correlation seems to exist between the rate of $NADPH_2$ oxidation by quinones and the $^{14}CO_2$-fixation rate. The effect of quinones on $NADPH_2$ oxidation and $^{14}CO_2$-fixation were in the order of Dichlone>06-K>NQ>BQ.

3. It is postulated that the phytotoxicity of quinones on *Chlorella* is due to the deprival of $NADPH_2$ consequently inhibiting $^{14}CO_2$-fixation, thus causing death of the cells.

4. The effect of quinones on amino acids biosynthesis in *Chlorella* was one of depressed rates, which

was especially noted in the case of dichlone. This would be expected from a consideration of $NADPH_2$ deprival and inhibition of $^{14}CO_2$-fixation. Sucrose synthesis was either not affected or rather stimulated, the reasons of which are not clear at the present time.

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