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INHIBITION OF PARTIAL REACTIONS IN BACTERIAL PHOTOSYNTHESIS BY 3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA

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SUMMARY

Inhibition by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) of the following partial reactions of bacterial photosynthesis has been examined using chromatophores prepared from light-grown *Rhodospirillum rubrum*: ascorbate- and PMS-induced photophosphorylation, NADH oxidation, NADH oxidatively coupled phosphorylation, NADH–cytochrome c_2 reduction, succinate–NAD^+ photoreduction, and anaerobic NADH oxidation by fumarate. All of these reactions were found to be inhibited by DCMU (and 3-(*p*-chlorophenyl)-1,1-dimethylurea) at concentrations in the 0.1 to 1.0 mM range. However, succinate–cytochrome c_2 reduction, NADH–2,6-dichlorophenolindophenol reduction and soluble NADH:cytochrome c_2 reductase were not inhibited. Based on these findings, it is proposed that DCMU and related compounds inhibit electron transport in chromatophores at a site(s) between NADH and either cytochrome *b* or a component on the reducing side of cytochrome *b*.

INTRODUCTION

Since the initial finding by WESSELS AND VAN DER VEEN[1] that 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU) and its derivatives inhibit photochemical reactions of chloroplasts, these compounds have been used extensively, particularly as specific inhibitors of O_2 evolution[2,3]. Researches on the mechanism of action have led to suggestions by GINGRAS, LEMASSON AND FORK[4] and by ASAHI AND JAGENDORF[5] that there could be at least two sites for inhibition. The inhibitory effect of CMU in a non-photosynthetic process (yeast respiration) has been reported by MUKASA, ITOH AND NOSOH[6], suggesting to them that the site of action is located between cytochrome *b* and cytochrome *c* in the electron-transfer chain. Such studies have not been extended to bacterial photosynthetic systems, apparently because of preoccupation with the notion that inhibition is confined to the typical green plant phenomenon of photosynthetic O_2 evolution, a process which does not occur in bacterial photosynthesis.

In this paper, we report briefly results of studies on the effects of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on photophosphorylation and associated electron-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol; BChl, bacteriochlorophyll; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide.

transfer reactions in chromatophores from light-grown, anaerobic cultures of *Rhodospirillum rubrum*.

MATERIALS AND METHODS

Chromatophores were prepared from light-grown cells of wild type and the blue-green mutant, G-9, of *R. rubrum* as reported previously[7] with the slight modification that cells were disrupted using a Serval Ribi cell fractionator, Model RF-1, at 20000 $lb \cdot inch^{-2}$, and thereafter purified by centrifugation.

Levels of photophosphorylation and oxidative phosphorylation activities were determined by ^{32}P incorporation into ATP, as described previously[8].

NADH- and succinate-cytochrome c_2 reduction were assayed by methods given in an earlier paper[9]. For 2,6-dichlorophenolindophenol (DCIP) reduction, 0.2 mM DCIP was substituted for cytochrome c_2, and the enzymatic activity was estimated by the absorbance change at 660 mμ.

Photoreduction of NAD^+ was assayed as described in a previous report[7]. Illumination with far-red light was effected by introducing the actinic beam through a port in the side of a Cary spectrophotometer, using an interference filter having a transmission peak at 878 mμ. The light intensity was $3.8 \cdot 10^4$ $erg \cdot cm^{-2} \cdot sec^{-1}$.

NADH oxidation by fumarate was anaerobically assayed with the following reaction components in 3 ml in a Thunberg-type cuvette: 50 mM Tris–HCl buffer, 5 mM $MgCl_2$, 10 mM sodium fumarate, 0.2 mM NADH, chromatophores (0.021 mM bacteriochlorophyll (BChl)) and water. The cuvette was alternately evacuated and flushed with argon and left under an atmosphere of argon. The difference in absorbance at 340 mμ between the sample and a reference cuvette without NADH was recorded in the dark. In the case of NADH oxidation, sodium fumarate was omitted from the reaction mixture and the assay conducted aerobically.

Cytochrome c_2 was prepared from light-grown cells of *R. rubrum* by the method of KAMEN *et al.*[10]. Throughout all experiments, Tris–HCl buffer (pH 7.2) containing 10 % sucrose was used. In experiment with DCMU and antimycin A, these reagents in methanol solution were added to the reaction mixture. The concentration of methanol used had no effect on the rates of these reactions.

DCMU and CMU were kindly supplied by Prof. W. L. BUTLER, Department of Biology in this University and Dr. CHAS. W. TODD of the Central Research Laboratory of E. I. Dupont, respectively. NADH, NAD^+, ATP, ADP and hexokinase were obtained from Sigma Chemical Co., St. Louis, Mo. ^{32}P-labeled P_i was purchased from New England Nuclear Co., Boston, Mass., and was used without further purification.

RESULTS AND DISCUSSION

First, we examined the effects of CMU and DCMU on ascorbate-induced photophosphorylation. Table I gives the results with DCMU. At low concentrations (< 20 μM), no inhibition was found. However, at higher concentrations, inhibition occurred (half-inhibition at 0.5 mM), much as in the inhibition of cyclic phosphorylation of chloroplasts and respiration in yeast[5,6]. CMU had much the same effect on photophosphorylation, being only slightly less effective at the observed concentration of 0.13 mM.

TABLE I

EFFECT OF DCMU AND ANTIMYCIN A ON PHOTOPHOSPHORYLATIONS INDUCED WITH ASCORBATE AND PMS

Experimental conditions were as described in text.

Inhibitor	*Concn.* (μM)	*Photophosphorylation induced by*			
		Ascorbate		*PMS*	
		Moles ATP formed/mole BChl/h	*Inhibition* (%)	*Moles ATP formed/mole BChl/h*	*Inhibition* (%)
DCMU	0	172	0	436	0
	20	169	2	445	2
	67	149	13	393	10
	200	115	33	318	26
	670	62	60	177	60
	2000	0.4	100	—	—
Antimycin A	2.4	2	99	430	1

Using phenazine methosulfate (PMS) to bypass the site of antimycin A inhibition (refs. 9, 11 and 12), we examined the effect of DCMU on PMS-induced photophosphorylation. The results are shown in Table I. In agreement with previous findings, 2.4 μM antimycin A inhibited ascorbate-induced photophosphorylation, but not PMS-induced photophosphorylation. In contrast, DCMU inhibited both processes to the same extent. Thus, we concluded that DCMU acted at a site(s) different from that binding antimycin A.

IZAWA *et al.*[13] and HIND AND OLSON[14] reached a similar conclusion in their work on chloroplasts. However, they used much lower concentrations of DCMU, so that the mechanisms of action in chloroplasts may not be identical to those in chromatophores. Similar conclusions have been reported by DUYSENS AND SWEERS[15].

Further confirmation of different sites for action of DCMU and antimycin A was obtained by recourse to spectrometric methods[16–19]. By such techniques, NISHIMURA AND CHANCE[17] succeeded in locating sites in the electron-transport system of photosynthetic bacteria for inhibition by antimycin A and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO).

Figs. 1 and 2 show the effects of antimycin A and DCMU on absorbance changes in the light *minus* dark difference spectra of chromatophores. In the absence of inhibitor, a minimum was observed in the difference spectrum at 556–558 mμ. This effect was reversible and corresponded to photo-oxidation of bound cytochromes c_2 and b. Upon addition of 24 μM antimycin A (Fig. 1c), a maximum at 563 mμ and a minimum at 551 mμ appeared.

In control experiments, it was found that reduction by ascorbate gave a reduced —oxidized difference spectrum with a peak at 551–552 mμ (characteristic of bound cytochrome c_2) and reduction by sodium dithionite gave an additional peak at 561–563 mμ (characteristic of cytochrome b). On the basis of these findings, the difference spectra obtained in the presence of antimycin A were interpreted as photo-oxidation of bound cytochrome c_2 and concomitant photoreduction of cytochrome b.

Accordingly, they indicate that light-driven electron flow was interrupted between cytochrome *b* and bound cytochrome c_2 in the presence of antimycin A.

The light *minus* dark difference spectrum in the presence of 1 mM DCMU (sufficient to inhibit 80 % of the ascorbate-induced photophosphorylation or NADH–cytochrome c_2 reduction) exhibited a minimum at 562–563 mμ (Fig. 2b). No other peaks were observed over a range from 540 to 580 mμ. Thus, only photo-oxidation of cytochrome *b* was indicated.

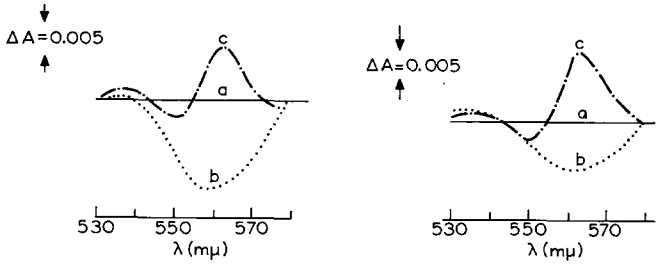

Fig. 1. Difference spectra, light *minus* dark, in the presence of antimycin A under aerobic conditions. Experimental procedure for illumination of light was the same as for succinate–NAD^+ photoreduction. Light intensity, $3.5 \cdot 10^{-4}$ erg·cm^{-2}·sec^{-1}; chromatophores (BChl), 0.09 mM; antimycin A, 24 μM. Chromatophores were prepared from light-grown blue-green mutant, G-9. Line a, base line; line b, no addition; line c, *plus* antimycin A.

Fig. 2. Difference spectra, light *minus* dark, in the presence of DCMU and antimycin A under aerobic conditions. Experimental conditions were the same as in Fig. 1. DCMU, 1 mM; line a, base line; line b, *plus* DCMU; line c, *plus* DCMU and antimycin A.

TABLE II

EFFECT OF DCMU ON NADH OXIDATION AND ITS COUPLED PHOSPHORYLATION

Experimental conditions were as described in text.

DCMU (mM)	*NADH oxidation*		*Oxidative phosphorylation*	
	Moles NADH oxidized/mole BChl/h	*Inhibition (%)*	*Moles ATP formed/mole BChl/h*	*Inhibition (%)*
0	39.1		9.0	
0.1	34.2	13	—	
0.3	—		4.9	45
0.5	21.4	45	—	
1.0	9.4	76	1.6	82

The further addition of 24 μM antimycin A caused the oxidation of bound cytochrome c_2 and reduction of cytochrome *b*, as with antimycin A alone. Therefore, one could conclude that DCMU did not block electron flow between bound cytochrome c_2 and *b*.

We have studied the effect of DCMU on the electron-transport components involved in NADH oxidation coupled to phosphorylation and described in previous researches[8,9,20]. As seen in Table II, DCMU inhibited NADH oxidation and its coupled phosphorylation. The degree of inhibition was almost the same as that for photophosphorylation (Table I). As noted in Table III, NADH–cytochrome c_2 reduction with chromatophores was markedly inhibited by DCMU and the extent of inhibition

TABLE III

EFFECTS OF DCMU AND ANTIMYCIN A ON NADH AND SUCCINATE CYTOCROME c_2 REDUCING ACTIVITIES WITH CHROMATOPHORES

Inhibitor	*Conc.* (μM)	*Reduced by*			
		NADH		*Succinate*	
		Moles cytochrome c_2 reduced/mole BChl/h	*Inhibition* (%)	*Moles cytochrome c_2 reduced/mole BChl/h*	*Inhibition* (%)
DCMU	0	137.0	—	10.6	
	10	137.0	0	—	
	100	121.2	2	—	
	300	97.8	29	—	
	500	68.4	50	—	
	1000	26.2	81	9.3	12
	1500	19.6	86	—	
Antimycin A	4.5	48.2	65	2.4	55
DCMU and antimycin A	4.5	27.4	80	—	

was the same as for ascorbate-induced photophosphorylation. 4.5 μM antimycin A, which is sufficient to inhibit completely ascorbate-induced photophosphorylation, did not totally inhibit this reducing activity. (A similar observation has been already reported by one of us[8].)

In an experiment using both 4.5 μM antimycin A and 1 mM DCMU, the inhibition of cytochrome c_2 reduction appeared to occur to the same degree with, or without, antimycin A. In contrast to NADH–cytochrome c_2 reduction, succinate–cytochrome c_2 reduction with chromatophores was not inhibited by DCMU, even at 1 mM, whereas this activity was diminished by the addition of 4.5 μM antimycin A (Table III). These observations are consistent with the supposition that the inhibition site of DCMU is localized in the photosynthetic cyclic electron-transport chain in chromatophores on the reducing side of cytochrome *b*.

HORIO AND KAMEN[21] isolated NADH: hemoprotein reductase from light-grown cells of *R. rubrum*. The same activity appeared in a supernatant solution obtained during a step in the purification of chromatophores, involving centrifugation at 144000 $\times g$ for 1 h. DCMU at 1 mM inhibited neither the soluble NADH: cytochrome c_2 reductase nor the same activity present in the supernatant solution. Also, NADH–DCIP reduction with chromatophores was not inhibited by 1 mM DCMU. From these results, we assume that the site of DCMU inhibition most probably involved some bound components of the chromatophores.

Having inferred that DCMU blocked electron flow between NADH and cytochrome *b*, we investigated its action on two other relevant enzyme systems. Thus, we found (Table IV) that both succinate–NAD^+ photoreduction and NADH–fumarate oxidation were inhibited by DCMU. Taken together with results of previous researches[8] which indicate that the NAD^+/NADH couple is not on the main path of cyclic electron transport, it appears reasonable to assert that DCMU acts at a site located between

TABLE IV

EFFECT OF DCMU ON SUCCINATE–NAD^+ PHOTOREDUCTION AND ON NADH OXIDATION BY FUMARATE

DCMU (μM)	*Photoreduction*		*Oxidation*	
	Moles NAD^+ reduced/mole BChl/h	*Inhibition* (%)	*Moles NADH oxidized/mole BChl/h*	*Inhibition* (%)
0	19.4		34.1	0
50	18.3	5	35.1	3
200	11.6	40	25.3	26
500	8.7	55	18.1	47

NADH and either cytochrome *b*, or some component on the reducing side of cytochrome *b* in the cyclic electron-transport system of chromatophores.

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