CYANIDE GENERATION FROM CARBONYLCYANIDE *m*-CHLOROPHENYLHYDRAZONE AND ILLUMINATED GRANA OR ALGAE

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1. Introduction

Nitrate reductase of *Chlorella vulgaris* has been shown to exist in an active and an inactive form which are readily interconvertible [1,2]. The inactive form contains bound cyanide which is released on activation [3]. In the course of a search for the conditions which lead to the inactivation of nitrate reductase in vivo, it was observed that the addition of the uncoupler, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to intact cells caused a rapid inactivation of the enzyme [4]. Carbonylcyanide phenyldrazone and its derivatives are known to be effective uncouplers of oxidative and photosynthetic phosphorylation [5–7]. At higher concentrations, these compounds also inhibit electron transport reactions [8].

The experiments reported here show that the addition of CCCP to *Chlorella vulgaris* or to spinach grana leads to the release of significant amounts of HCN, and that the cyanide release is stimulated by light and O_2. These results explain the rapid inactivation of nitrate reductase observed when CCCP is added to intact cells.

2. Materials and methods

2.1. *Reagents*

Carbonylcyanide.*m*-chlorophenylhydrazone (CCCP) was purchased from Serva, Heidelberg. Atebrin and undecylbenzimidazole [9] were generous gifts from Professor Dr A. Trebst, Ruhr-Universität Bochum.

2.2. *Biological materials*

Chlorella vulgaris cells were grown in continuous white light on mineral salts medium, pH 4.3, with nitrate as the only source of nitrogen, in a stream of 5% (v/v) CO_2 in air, at 20–22°C, as previously described [10,11]. Cells were harvested by centrifugation after 48 h.

For preparation of the French press extract, the cells were washed with 10 mM potassium phosphate buffer, pH 7.6, and resuspended in the same buffer to a concentration of 250 µl cells per ml. The cell suspension was disrupted with a French Pressure Cell Press (Aminco), precooled in ice, at 10 000 p.s.i. The broken cell suspension was centrifuged at 10 000 *g* for 10 min, and the decanted supernatant was stored in an ice bath till used.

Grana were prepared from *Spinacea oleracea* (Matador). Freshly picked leaves were washed and the midribs were removed. The leaves were ground with water (20 ml per 20 g leaves) in a blender (Janke and Kunkel, Staufen i.Br., type A 10) twice for 15 sec, with cooling. The slurry was pressed through cheese cloth and centrifuged at 1000 *g* for 2 min. The supernatant was decanted and centrifuged at 35 000 *g* for 20 min. The resulting pellet was washed once with 40 ml distilled water, sedimented again by centrifugation, and suspended in distilled water to give a chlorophyll concentration of 1 mg per ml.

2.3. *Experimental procedure and measurement of HCN*

The experiments were carried out in Warburg vessels with one side arm and a center trough. The

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vessels were connected to manometers and shaken in a Warburg bath with a glass bottom. The temperature was kept at 20°C. Illumination was provided by 6 Comptalux bulbs, each 150 W, mounted in two rows below the bath. Each row was cooled by an air blower. The bottom of the bath was covered with red cellophane. The illumination intensity, measured 3 cm above the water surface with a Panlux electronic (Gossen), gave a value of approx. 9000 lux. When O_2 or argon were employed, the vessels were gassed for 5 min prior to the start of illumination. The suspension of biological material was placed in the main compartment of the vessel, and additions were made as indicated. The central trough contained 0.2 ml of 0.5 N NaOH, to absorb the HCN released. Unless otherwise indicated, the vessels were shaken for a period of three hours. HCN was determined on aliquots of the alkali in the center trough, by the method of Guilbault and Kramer [12]. Because the HCN has a catalytic effect in this method, the sensitivity can be increased by prolonging the incubation time. For the lower HCN concentrations, samples were incubated for 1 h at 20°C. Otherwise shorter periods were used. Calculations were made from standard curves performed in identical fashion.

Table 1
Recovery of added cyanide from intact cells of *Chlorella vulgaris* or spinach grana

	Cyanide added (nmoles)	Cyanide recovered (nmoles)	(%)
Intact cells of Chlorella vulgaris	200	134.0	67
	20	12.0	60
	5	2.9	58
	1	0.6	60
Spinach grana	200	180.0	90
	20	15.3	77
	5	3.8	76
	1	0.8	80

The Warburg vessels contained 3 ml of *Chlorella* suspension (conditions as in table 2) or 0.1 ml grana in potassium phosphate buffer, pH 7.0 (conditions as in table 4). 0.05 ml KCN solution of indicated concentration in 1 mM NaOH was pipetted into the side arm of the Warburg vessel and tipped after the vessel was connected with a manometer. The Warburg vessels were shaken in light and in air.

Essentially all (95%) of the HCN added to buffer, in the absence of cells or grana, is recovered in the alkali after 3 h of shaking in the dark. In the presence of cells or grana, however, recovery of added cyanide is incomplete. Table 1 shows the recovery observed when different amounts of HCN were added to cells or grana, under the conditions employed in the experiments of tables 2 and 4 respectively, in the light in air.

In addition to incomplete recovery, the method described has the shortcoming that the process of HCN generation and collection have not been separated. Thus the cyanide generated in the third hour will in part be uncollected, in comparison with the cyanide generated in the first hour. Furthermore, with longer incubation periods, broken cell preparations give off other volatile substances which accumulate in the alkali and react with the cyanide, diminishing the yield.

In spite of these obvious flaws, we believe that the amounts of HCN found have comparative significance. Reproducibility is good. So far as we know, only HCN or a volatile cyanogen will give a positive test with the procedure described.

3. Results

3.1. *Experiments with whole cells of Chlorella vulgaris*

In the experiments of table 2, *Chlorella* cells suspended in mineral salts solution, were placed in the main compartment of Warburg vessels, with alkali in the center well, and shaken for three hours under the various conditions indicated. There was no detectable cyanide released from *Chlorella* cells alone; and there was likewise no cyanide released from CCCP alone, under the conditions here employed. When, however, CCCP was added to *Chlorella* cells, cyanide was released into the gas phase and absorbed by the alkali. The amount of cyanide collected increased with increasing amounts of added CCCP. There was more cyanide collected in the light than in the dark, and O_2 had a stimulatory effect.

3.2. *Experiments with broken cells of* Chlorella

The behavior of broken cells was rather similar to that of intact cells, except that smaller amounts of HCN were obtained from broken cells alone, without

Table 2
Release of cyanide from intact cells of *Chlorella vulgaris* and CCCP

Additions	Light/dark	Gas phase	HCN found (nmoles)
5×10^{-5} M CCCP, alone	Light	Air	0
1×10^{-5} M CCCP, alone	Light	Air	0
Chlorella, alone	Light	Air	0
Chlorella, 1×10^{-5} M CCCP	Dark	Air	0.1
Chlorella, 5×10^{-5} M CCCP	Light	Air	45.6
Chlorella, 1×10^{-5} M CCCP	Light	Air	3.6
Chlorella, 5×10^{-6} M CCCP	Light	Air	2.2
Chlorella, 1×10^{-6} M CCCP	Light	Air	0.1
Chlorella, alone	Light	O_2	0.1
Chlorella, 1×10^{-5} M CCCP	Light	O_2	5.2
Chlorella, alone	Light	Air	0
Chlorella, 1×10^{-5} M CCCP	Light	Air	3.6
Chlorella, alone	Light	Argon	0
Chlorella, 1×10^{-5} M CCCP	Light	Argon	0.8

Chlorella cells were washed with nitrate-free salts solution, pH 4.3, and suspended in the same solution to a concentration of 7 μl cells per ml. 3 ml of this solution were added to the main compartment of the Warburg vessels. Other additions were as indicated. The vessels without cells contained 3 ml of nitratefree salts solution and CCCP as indicated. For further details, see Materials and methods.

added HCN or other reagents (table 3). Far more HCN was obtained when CCCP was added. Other uncouplers of photosynthetic phosphorylation, like atebrin or undecylbenzimidazole did not increase the HCN yield like CCCP.

Table 3
Release of cyanide from French press extract of *Chlorella vulgaris*

Additions	HCN found (nmoles)
None	0.8
5×10^{-5} M CCCP	40.4
1×10^{-5} M CCCP	6.8
5×10^{-6} M CCCP	3.2
1×10^{-5} M Atebrin	0.6
1×10^{-5} M Undecylbenzimidazole	0.4

Each vessel contained 1 ml of broken cell preparation (French press), 150 μmol of potassium phosphate buffer, pH 7.0, and 50 μmol KCl in a total vol of 3 ml. Other additions were as indicated. The Warburg vessels were shaken in light and in O_2 atmosphere. For further details, see Materials and methods.

3.3. *Experiments with spinach grana*

The results obtained with spinach grana (table 4) were similar to those with broken *Chlorella* cells except that the yields of HCN were higher. The amounts of HCN collected were roughly proportional to the amounts of CCCP added. Light and O_2 had strong stimulatory effects. Undecylbenzimidazole or atebrin did not cause any release of HCN. Under the conditions here employed, at pH 7.0, CCCP alone gave a small amount of HCN, although no HCN was obtained from CCCP alone at pH 4.3 (table 2).

The results given in table 5 show the course of the cyanide collection with time, from CCCP and spinach grana. These measurements were made with 5×10^{-5} M CCCP in O_2 in light. The maximum yield was obtained in 3 h and amounted to 0.8 mole per mole of CCCP added.

4. Discussion

On introducing carbonylcyanide phenylhydrazone and its derivatives as potent uncouplers, Heytler,

Table 4
Release of cyanide upon addition of CCCP or other uncouplers to spinach grana

Additions	Light/dark	Gas phase	HCN found (nmoles)
5×10^{-5} M CCCP, alone	Light	O_2	0.5
1×10^{-5} M CCCP, alone	Light	O_2	0.2
5×10^{-5} M CCCP, alone	Light	Air	0.2
1×10^{-5} M CCCP, alone	Light	Air	0
Grana, alone	Light	Oxygen	0.2
Grana, 5×10^{-5} M CCCP	Dark	Air	0.9
Grana, 5×10^{-5} M CCCP	Light	Argon	9.7
Grana, 5×10^{-5} M CCCP	Light	Air	91.0
Grana, 5×10^{-5} M CCCP	Light	Oxygen	144.2
Grana, 1×10^{-5} M CCCP	Light	Oxygen	58.8
Grana, 5×10^{-6} M CCCP	Light	Oxygen	16.0
Grana, 1×10^{-6} M CCCP	Light	Oxygen	2.2
Grana, 5×10^{-5} M Atebrin	Light	Oxygen	0.3
Grana, 5×10^{-5} M Undecylbenzimidazole	Light	Oxygen	0.2

The main compartment of each vessel contained 0.1 ml grana (0.1 mg chlorophyll), 150 μmol of potassium phosphate buffer, pH 7.0, and 50 μmol KCl in a total vol of 3 ml. Other additions were as indicated. The vessels without grana contained 150 μmol potassium phosphate buffer, pH 7.0, and CCCP as indicated in a total vol of 3 ml. For further details, see Materials and methods.

Heytler and Prichard [5,6] suggested that the dicyanomethylene groups might have a direct function in the uncoupling. The experiments here described show that considerable quantities of HCN are liberated when CCCP is incubated with Chlorella or spinach grana. It seems likely that most of this HCN has its origin in the cyano group(s) of the uncoupler. Further experiments are necessary to determine whether the HCN generation can account for any of the properties of CCCP as an uncoupler and, at higher concentrations, an inhibitor of redox reactions.

Table 5
Time course of cyanide release from CCCP in the presence of grana

Period of illumination (hours)	HCN found (nmoles)
1	17.2
2	47.5
3	114.0
4	113.0

Conditions were as in table 4. The Warburg vessels were shaken in light and O_2 atmosphere for the times indicated. The concentration of CCCP was 5×10^{-5} M.

The active generation of HCN on addition of CCCP explains the observation that nitrate reductase is rapidly and reversibly inactivated when small amounts of CCCP are added to *Chlorella* cells. This inactivation requires only minute amounts of HCN. It has previously been shown that HCN can be obtained from both *Chlorella* and spinach [13], and the present data confirm this. Only very small amounts of HCN are formed in the absence of added cyanogens. The natural precursor(s) of this HCN is not yet known. The present observations may provide a clue to the biogenesis process, in the sense that CCCP may be taken as a model for the natural precursor. In support of this is the fact that the formation of HCN in *Chlorella* in vivo, as monitored by the formation of reversibly inactivated nitrate reductase [4], is stimulated by light and high O_2 tension, though it also occurs anaerobically in the dark. The generation of HCN on CCCP addition shows these same characteristics.

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