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THE EFFECTS OF 2,4-DINITROPHENOL AND OTHER UNCOUPLING AGENTS ON THE ASSIMILATION OF NITRATE AND NITRITE BY CHLORELLA

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SUMMARY

1. 2,4-Dinitrophenol and five other uncoupling agents (at concentrations of 1.0 mM) inhibit the utilization of nitrate by about 80–95 %. The inhibitions can be relieved by increasing nitrate concentrations.

2. The uncoupling agents fail to inhibit the activity of nitrate reductase in cell-free extract.

3. Inhibition of nitrite utilization by the six uncoupling agents depends in the experimental conditions. Under heterotrophic conditions, all six inhibit. When cultures are illuminated (particularly in the absence of CO_2) three of the uncouplers are much less effective as inhibitors than they are under heterotrophic conditions.

INTRODUCTION

Observations by KESSLER[1,2], have suggested that reduction of nitrite by green alga *Ankistrodesmus braunii* is inhibited by the uncoupling agent 2,4-dinitrophenol, whereas reduction of nitrate is insensitive to the inhibitor. The latter conclusion was based on the observation that nitrite accumulated in the medium when the alga was incubated in the presence of nitrate and 2,4-dinitrophenol. Comparable inhibition by 2,4-dinitrophenol has also been observed for Azotobacter[3] and for Anabaena[4]. KESSLER AND CZYGAN[5] also observed that the uncoupling agent inhibited the activity of nitrite reductase (NAD(P)H:nitrite oxidoreductase, EC 1.6.6.4) extracted from Ankistrodesmus, but had no effect on the activity of nitrate reductase (NAD(P)H:nitrate oxidoreductase, EC 1.6.6.2) activity in cell-free extract. The activity of nitrite reductase was also inhibited by arsenate[6], and the dinitrophenol inhibition could be reversed by ATP. Comparable inhibition of nitrite-reductase activity by 2,4-dinitrophenol and arsenate has also been observed with extracts from *Anabaena cylindicrica*[7].

On the basis of the above observations it has been inferred that the reduction of nitrite by these organisms requires 'high-energy' phosphate compounds, whereas the reduction of nitrate to nitrite has no such requirements. The nature of the energy requirement and the precise mechanism of inhibition by 2,4-dinitrophenol require clarification; particularly in view of the fact that the photochemical reduction of

nitrite by chloroplast fragments is accompanied by the synthesis of ATP and not its utilization[8–10].

Preliminary observations in this investigation have shown that the utilization of nitrate by Ankistrodesmus is not completely insensitive to 2,4-dinitrophenol[11]. These preliminary observations are extended in this present study on the green alga Chlorella, and an attempt is made to understand the precise nature of the inhibition of nitrate and nitrite utilization by uncoupling agents.

MATERIALS AND METHODS

Growth of organism. Pure cultures of *Chlorella pyrenoidosa* Strain 211/8p were maintained and grown as described by SYRETT AND MORRIS[12] (the name of the organism has been recently changed from *Chlorella vulgaris*[13]).

General experimental procedure. After 4 days growth the organism was harvested by centrifugation, washed once with N-free medium (full growth medium *minus* the N source) and resuspended in this medium. The cell density was adjusted turbidometrically to 3–8 mg dry weight per ml depending on the experiment.

Estimation of nitrate-N, nitrite-N and ammonium-N. Utilization of these N sources was measured by following their disappearance from the medium. Nitrate-N was determined by a phenoldisulphonic acid colorimetric method[14], and nitrite-N was determined colorimetrically with GRIESS–ILOSVAY reagents[14] (further details are given in SYRETT AND MORRIS[12]). Ammonium-N was determined colorimetrically by Nesslerization[15] following the micro-diffusion technique of CONWAY[16].

Determination of nitrate-N in the presence of organo nitro-compounds. 2,4-Dinitrophenol and the other organic nitro-compounds used in this investigation interfere with the assay of nitrate-N. The interfering compounds were removed from the sample by adsorption on to activated charcoal[14].

Gas exchange. This was measured by conventional WARBURG manometry at 25°. For appropriate experiments the cultures were illuminated with fluorescent tubes giving a light intensity of about 800 ft-candles at the culture surface. For measurements of hydrogenase activity, the flasks were shaken in the dark in an atmosphere of H_2 for 4 h before addition of the appropriate N-compound. Preliminary experiments established that this pre-incubation period gave maximal rates of H_2 uptake during the subsequent experimental period.

Assay of nitrate reductase activity. Cell-free extracts were prepared as described previously[12] and nitrate-reductase activity was measured as described by MORRIS AND SYRETT[17]. Preliminary attempts to measure enzymatic reduction of nitrite by cell-free extracts from Chlorella (using various combinations of benzyl viologen, sodium dithionite and NADPH) have so far failed.

RESULTS

Inhibition of nitrite utilization by 2,4-dinitrophenol

Fig. 1 shows the effect of 1.0 mM 2,4-dinitrophenol on the utilization of nitrite by Chlorella; the inhibition resembles that observed by KESSLER[1,2] for Ankistrodesmus. Nitrite assimilation was measured under two conditions; either in darkness in the presence of glucose (Fig. 1a) or in the light in the presence of CO_2 (Fig. 1b)

The degree of inhibition under the latter conditions appears to be less than under the former (70% compared with 100%).

Effect of 2,4-dinitrophenol on nitrate utilization

Because nitrite accumulated in the medium when Ankistrodesmus cultures were incubated with nitrate and 2,4-dinitrophenol, KESSLER concluded that the reduction of nitrate to nitrite was insensitive to the inhibitor. However, the rate of nitrite accumulation in Chlorella cultures supplied with nitrate and 2,4-dinitrophenol is significantly less than the rate of nitrate utilization in the absence of the uncoupling agent. This suggests that the utilization of nitrate is not completely insensitive to 2,4-dinitrophenol. Direct measurements of the effect of 2,4-dinitrophenol on nitrate disappearance confirms this (Fig. 2b). Fig. 2 also shows that the degree of inhibition of nitrate utilization by 2,4-dinitrophenol is dependent on the concentration of nitrate (this confirms the earlier observation from this laboratory with Ankistrodesmus[11]). The dependence of the degree of inhibition on the nitrate concentration is shown by measurements of nitrate disappearance (Fig. 2b) and by measuring accumulation of nitrite (Fig. 2a).

Effect of several uncoupling agents on the utilization of nitrate and nitrite by Chlorella

Table I shows the effect of six uncoupling agents on nitrate and nitrite utilization. At concentrations of 1.0 mM all six inhibit nitrate utilization (their uncoupling properties were confirmed by observing inhibition of [^{14}C]glucose incorporation into the alcohol-insoluble fraction of Chlorella by comparable concentrations of the uncoupling agents). Although inhibition of nitrate utilization varies from 64–98%, the pattern of inhibition is the same in both heterotrophic and illuminated cultures. However, the degree of inhibition of nitrite utilization by certain of the inhibitors

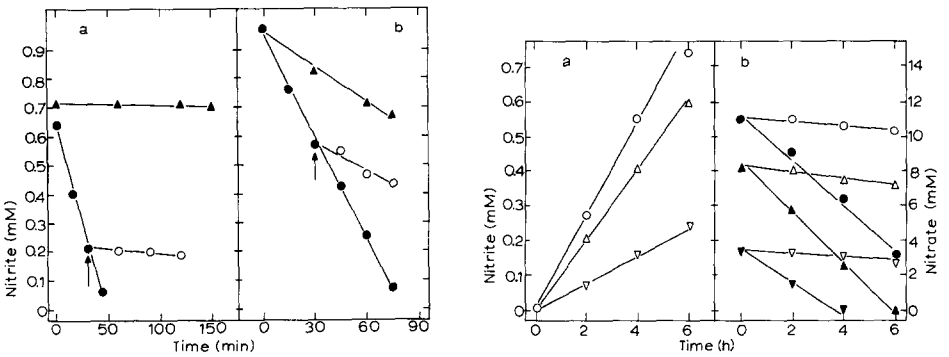

Fig. 1. The effect of 2,4-dinitrophenol on nitrite utilization by cultures of Chlorella incubated in (a) darkness + 1% (w/v) glucose and (b) light + 5% CO_2 in air. Nitrite disappearance was followed in the absence of 2,4-dinitrophenol (●—●) and in cultures supplied with 1.0 mM 2,4-dinitrophenol added at zero time (▲—▲) and at the time indicated (○—○). Cell density, 3.0 mg dry wt./ml; temp., 25°.

Fig. 2. The effect of 2,4-dinitrophenol (1.0 mM) on (a) nitrite accumulation and on (b) nitrate disappearance at three nitrate concentrations. The initial nitrate concentrations were 10.36 mM (●—●, ○—○) 7.85 mM (▲—▲, △—△) and 3.57 mM (▼—▼, ▽—▽). The black symbols show nitrate disappearance in the absence of 2,4-dinitrophenol and the white symbols show nitrate disappearance and nitrite accumulation in its presence. Cultures (density, 8 mg dry wt./ml) were incubated at 25° in darkness in the presence of 1% glucose.

depends on the experimental conditions under which nitrite utilization is measured. When cultures are illuminated in the absence of CO_2, the three dinitrophenols inhibit nitrite utilization by 77–86%, whereas *p*-nitrophenol, *o*-nitrophenol and 2,4-dichlorophenol inhibit by only 24–27%. Thus, under these conditions three uncoupling agents inhibit nitrite utilization significantly less than do the remaining three. This dichotomy is less marked when cultures are illuminated in the presence of CO_2 and disappears completely when cultures are incubated under heterotrophic conditions.

TABLE I

THE EFFECT OF SELECTED ORGANO NITRO- (AND CHLORO-) COMPOUNDS ON THE RATE OF NITRATE AND NITRITE REDUCTION

For measurements of nitrate and nitrite assimilation cultures (cell density, 3.0–5.0 mg dry wt./ml) were incubated in darkness in the presence of 0.5% glucose, or illuminated either in flasks containing 10% KOH in centre wells or bubbled with a slow stream of 5% CO_2 in air. The initial nitrate concentration was 5 mM and that of nitrite was 1.0 mM.

Compounds added (1.0 mM)	*Percentage inhibition of nitrite assimilation*			*Percentage inhibition of nitrate assimilation*		*Effect on photophosphorylation*[18]
	Light $-CO_2$	*Light* $+CO_2$	*Dark* $+glucose$	*Light* $-CO_2$	*Dark* $+glucose$	
2,4-Dinitrophenol	77	75	100	75	98	Active
2,5-Dinitrophenol	82	84	76	75	86	Active
2,6-Dinitrophenol	86	64	70	60	70	Active
p-Nitrophenol	25	50	83	100	77	Active
o-Nitrophenol	24	43	54	100	88	Inactive
2,4-Dichlorophenol	27	43	77	100	64	Slightly active

The final column of Table I presents the results of WESSELS[18] who investigated the effect of the same six organo nitro- (and chloro-) compounds on photophosphorylation by an isolated chloroplast system. With the exception of *p*-nitrophenol, there is correlation between inhibition of nitrite utilization in illuminated cultures of Chlorella and the ability of the same compounds to serve as catalysts of photophosphorylation in the experiments of WESSELS.

Attempts to demonstrate that the same three dinitrophenols which inhibit nitrite reduction by cultures illuminated in the absence of CO_2 are also reduced under the same conditions have not yielded unequivocal results.

The effect of organo nitro- (and chloro-) compounds on nitrate reductase activity and on hydrogenase-mediated nitrite reduction

Although the utilization of nitrate by intact cells of Chlorella is inhibited by all six uncoupling agents used in this investigation, the same concentration (1.0 mM) of these compounds does not inhibit the activity of nitrate reductase in cell-free extracts from Chlorella.

Comparable experiments with nitrite reductase in cell-free extracts cannot be undertaken since it has not yet been possible to detect any nitrite-reductase activity in extracts from this strain of *Chl. pyrenoidosa*. Instead, the effect of the uncoupling agents on hydrogenase-mediated nitrite reduction by intact cells has been studied, since it is possible that the reduction of nitrite by H_2 *via* a hydrogenase system (such

a complete sequence has been demonstrated by PANEQUE *et al.*[19]) might afford a means of studying the effects of the inhibitors on nitrite reduction under conditions when the latter is relatively isolated from other areas of metabolism.

TABLE II

THE RATES OF NITRITE REDUCTION AND OF HYDROGEN CONSUMPTION IN THE PRESENCE OF SELECTED ORGANO NITRO- (AND CHLORO-) COMPOUNDS

Cultures (3 mg dry wt./ml) were incubated in darkness and the nitrogen compounds (1.0 mM) added after a 4-h preincubation period in an atmosphere of H_2. Uptake of H_2 was followed (and was linear) for 1 h, and the amount of nitrite which disappeared during that time was also determined.

Compound added	*Rate of nitrate reduction (μmoles nitrite/mg cells/h)*	*Rate of H_2 consumption (μmoles H_2/mg cells/h)*
None	—	0.05
Nitrite	0.27	1.70
Nitrite + 2,4-dinitrophenol	0.14	0.53
Nitrite + 2,5-dinitrophenol	0.18	0.90
Nitrite + 2,6-dinitrophenol	0.18	0.90
Nitrite + *o*-nitrophenol	0.27	1.70
Nitrite + *p*-nitrophenol	0.27	1.53
Nitrite + 2,4-dichlorophenol	0.27	1.17
2,4-Dinitrophenol	—	0.38
2,5-Dinitrophenol	—	0.18
2,6-Dinitrophenol	—	0.40
o-Nitrophenol	—	0.59
p-Nitrophenol	—	0.13
2,4-Dichlorophenol	—	0

Table II summarizes the effect of the six organo nitro- (and chloro-) compounds on the uptake of hydrogen which accompanies the reduction of nitrite by cells possessing hydrogenase activity. The three dinitrophenols which inhibit nitrite reduction by cultures illuminated in the absence of CO_2 (Table I) also show the most severe inhibition of hydrogen uptake. Also analysis of the initial and final concentrations of nitrite confirm that the same three dinitrophenols inhibit the disappearance of nitrite most markedly. However, the degree of inhibition of nitrite disappearance in these experiments is less than in cultures illuminated in the absence of CO_2 and is also less than the inhibition of hydrogen uptake (the three dinitrophenols inhibit hydrogen consumption by 64–77% but inhibit nitrite disappearance by only 44–48%).

Stimulation of hydrogen uptake by the organo nitro- (and chloro-) compounds (added separately) is also shown in Table II.

The rate of hydrogen absorption in the absence of added compounds is about 0.05 μmole of hydrogen per mg cells per h, and in the presence of nitrite is about 1.70 μmoles of hydrogen per mg cells per h. That is, it does not appear that all the additional hydrogen absorbed in the presence of nitrite is used for nitrite reduction. The effect of the added compounds on hydrogen consumption and on accompanying nitrite reduction is therefore difficult to evaluate. Paper chromatographic analyses show that the reduction products can be detected only in those flasks supplied with the dinitrophenols alone, and not when these compounds are added together with nitrite.

DISCUSSION

Contrary to earlier ideas the utilization of nitrate by Chlorella (and also by Ankistrodesmus[11]), is not completely insensitive to 2,4-dinitrophenol. Rather the utilization of nitrate by these algae is inhibited by 1.0 mM 2,4-dinitrophenol by about 80–95%. Since inhibition of nitrite utilization is generally greater than this, nitrite accumulates in the medium of cultures incubated with nitrate and 2,4-dinitrophenol (described earlier by KESSLER[1,2]).

Although the six uncoupling agents used in this present investigation inhibit the utilization of nitrate by intact cells of Chlorella, they have no effect on the activity of nitrate reductase in extracts from the same organism. Possibly, the inhibitors prevent the entry of nitrate into the cells and not its reduction to nitrite. Several observations on absorption of nitrate by fungal cells[20] and by cells of higher plants[21] support the suggestion that nitrate is absorbed by both a passive, dinitrophenol-insensitive process, and an active, energy-requiring process. The relief of inhibition by increasing nitrate concentration observed in this paper might be due to the passive process increasing in importance with increasing nitrate concentration. It has not been possible to test this hypothesis directly since nitrate cannot be detected in the cells of either Chlorella or Ankistrodesmus.

It is unlikely that inhibition of nitrite utilization is due to inhibition of the absorption of nitrite. It is probable that nitrite enters Chlorella as undissociated nitrous acid[22], and absorption of undissociated molecules appears to be less dependent on energy than does that of ions[23]. Also, KESSLER AND CZYGAN[5] observed inhibition by 2,4-dinitrophenol of nitrite-reductase activity in extracts from Ankistrodesmus, and arguments by analogy with the observations of KESSLER AND CZYGAN would support the suggestion that inhibition by uncoupling agents of nitrite utilization in the present investigation is due to an inhibition of nitrite reduction.

Nitrate utilization shows the same sensitivity to the uncoupling agents in cultures incubated in darkness in the presence of glucose as it does in cultures illuminated in the absence of CO_2. Nitrite utilization, however, is less sensitive to three of the uncoupling agents in cultures illuminated in the absence of CO_2 than it is in heterotrophic cultures.

The ability of the three dinitrophenols to inhibit nitrite reduction by cultures illuminated in the absence of CO_2 might be related to the ability of the inhibitors to become reduced under the same conditions. Although WESSELS[24] observed a non-enzymatic reduction of organo nitro-compounds by reduced ferredoxin, we have not obtained unequivocal evidence of reduction of these compounds by Chlorella, particularly at rates which would allow them to compete with nitrite for available reductants.

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ZUSAMMENFASSUNG

Es wird eine Methode zur Isolierung und Reinigung von Mitochondrien aus verschiedenen Entwicklungsstadien von Königinnen und Arbeiterinnen der Honig-

biene beschrieben. Durch Zentrifugation der rohen Mitochondrienfraktion in einem flachen Saccharose-Gradienten, der gleichzeitig einen steilen Gradienten von Stractan AF/2 (Meyhall) enthält, wird ein Mitochondrienpräparat erhalten, welches frei von Sarkosomen, Lysosomen und von Mikrosomen ist. Die Zellpartikel wurden durch Bestimmung des Protein- und RNA-Gehalts und der Aktivitäten von saurer Phosphatase, saurer Ribonuclease, Uricase und Succinatdehydrogenase charakterisiert. Das Sedimentationsprofil zeigt drei Proteinmaxima, von denen das mittlere die Mitochondrien, das leichtere Maximum die Lysosomen enthält. Der Mitochondrienbereich trennt sich bei Larven und weissäugigen Puppen in eine schwere und eine leichte Fraktion auf. Die Mitochondrienmenge ist in allen Entwicklungsstadien der Königinnen im Vergleich zu entsprechenden Arbeitsbienen mehr als doppelt so hoch. Bei Atmungsmessungen zeigen die Substrate Succinat, α-Glycerophosphat und Butyrat ein Aktivitätsmaximum in den Mitochondrien fünftägiger Larven. Ihre Aktivität ist besonders während der Larvenentwicklung in den Königinnen wesentlich höher als in den Arbeitsbienen. Ein Vergleich der Differenzspektren zeigt bei beiden Kasten in den frühen Entwicklungsstadien einen Mangel an Cytochrom *c*. Arbeiterinnenlarven enthalten im Vergleich zu gleichaltrigen Königinnenlarven weniger Cytochromoxydase und weniger Cytochrom *c*. Unter der Wirkung der im Weiselzellenfuttersaft enthaltenen determinierenden Substanz wird offenbar in der Königinnenlarve das Mitochondrienwachstum stimuliert und das Cytochromsystem optimal aufgebaut. In der Arbeiterinnenlarve führt die geringere Mitochondrienmenge zusammen mit einer verringerten Aktivität der Atmungskette dazu, dass der spezifische Sauerstoffverbrauch nur halb so hoch wie bei der Königinnenlarve ist.

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