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Azide Sensitivity in Species of Chlamydomonas and Carteria

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AZIDE SENSITIVITY IN SPECIES OF CHLAMYDOMONAS AND CARTERIA

(TITLE)

BY

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THESIS

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INTRODUCTION

Chlamydomonas and Carteria are two closely related unicellular genera of green algae (division Chlorophyta, order Volvocales, family Chlamydomonadaceae). Species of these and many other unicellular genera are often difficult to identify precisely without prolonged life history studies because of morphological variations induced by varying ecological circumstances. Carteria, for example, may be confused with the zoospores of Ulotrichalean algae while Chlamydomonas can be mistaken for a gamete or zoospore of many other algal species, such as Chlorococcum. Only through extensive laboratory studies under controlled environmental and physiological conditions can many unicellular algal isolates be correctly identified to genus and species.

Many morphological and physiological studies involving species of Chlamydomonas have been done in the past as evidenced by the excellent summation of pertinent literature by Cain (1963). His summation, however, fails to indicate whether or not vigorous axenic conditions were used by the various investigators in their physiological studies of Chlamydomonas.

The need for axenic cultures in any phycological experiment involving physiological parameters evolved from the careful considera-

tion given in bacteriology to axenic cultures for identification and other purposes. Bacterial contamination, in phycological studies, can and does lead to inaccurate conclusions as to the physiological characterization of a given algal species since bacteria may be the cause of various physiological observations that are attributed to that algal species. For example, Smith and Bold (1966), after careful preparation of axenic cells of Eremosphaera viridis, found that bacteria provided the vitamins (B_{12} and thiamine) necessary for growth of that alga. Thus, to insure that all physiological processes are attributed only to the algal species in question, axenic conditions must be maintained vigorously at all times.

The requirement for axenic cultures in physiological studies of unicellular and other algal species gave rise to the use of bacteriological techniques in algal taxonomy. Prescott (1964), Pringsheim (1967), Bold (1970), Dixon (1970), and Smith (1971) all point out the need for more physiological studies which can lead to attributes useful in algal taxonomy. Bold (1970) citing Pringsheim (1967), places particular emphasis on the importance of utilizing known bacteriological techniques in the taxonomy of morphologically similar unicellular algae. Such applications should, in theory, help clarify the present state of algal taxonomy in such genera as Chlamydomonas, of which over 500 species are presently described (Dixon, 1970).

In bacteriology, incorporation of specific compounds into en-

richment or selective media to isolate given genera is common. Such a compound is sodium azide (NaN_3) used to isolate Streptococcus (Keilin, 1936; Malmann, 1937). Sodium azide inhibits aerobic respiration (the cytochrome system) which is lacking in all members of the genus Streptococcus.

The literature is replete with investigations of sodium azide on living systems. In lieu of a detailed discussion of the plethora of reports on the affects of azide in living systems, the more important aspects found in the literature have been summarized in Table 1. The discussion which follows is concerned with the best known means of azide inhibition, viz., (1) azide inhibition of respiration via the cytochrome chain and (2) azide inhibition of substrate level and respiratory chain-phosphorylation.

It is well known that azide inhibits the passage of electrons through the cytochrome system. Figure 1 shows the reported site of azide inhibition in the cytochrome system as proposed by Yonetani and Ray (1965) in beef heart. From the diagram it is apparent that azide blocks the re-oxidation of cytochrome a, thus preventing O_2 from acting as the final electron acceptor in the chain. Azide, by holding cytochrome a in the reduced form, is a very effective inhibitor of cellular respiration.

While the locus of azide inhibition of respiration is generally agreed to be at the cytochrome a site in the electron transport system,

TABLE 1

Summary of Azide in the Literature

Inhibition involving electron transport and cytochrome a

Tissue type or Organism	Author(s)
horse liver	Blaschko, 1935
rat muscle oxidase	Chance, 1952
rat and guiena pig liver mitochondria	Chance & Williams, 1956
Prototheca zopfii	Katznelson & Stevenson, 1956
soil microfora	Keilin, 1936
horse liver	Keilin & Hartree, 1934, 1936, 1939
frog muscle	Stannard, 1939
rat liver mitochondria	Wilson, 1967
rat liver mitochondria	Wilson & Chance, 1967
beef heart	Yonetani & Ray, 1965

Inhibition of ATPF ormation

rat and guiena pig liver mitochondria	Chance & Williams, 1956
rat liver mitochondria	Dawkins & Judah, 1960
beef heart mitochondria	Fyfe, Bygrave, Lehninger, 1966
corn mitochondria	Hodges & Elzam, 1967
frog kidney homogenate	Loomis & Lipman, 1949
chicken embryos	Meyerhof & Wilson, 1949
rat liver mitochondria	Myers & Slater, 1957
rat liver mitochondria	Nicholls & Kimelberg, 1968
rat liver mitochondria	Ninneman, 1970
cat, pig and rat heart sarcosomes	Slater, 1955
rat mitochondria	Tsujmoto & Kawaguchi, 1962
rat mitochondria	Wadkins & Lehninger, 1958, 1963

Inhibition of photosynthesis and/or algae

chard chloroplasts	Arnon & Whatley, 1949
hexokinase uptake in Chlorella vulgaris	Decker & Tanner, 1972
Chlorella ellipsoidea	Hiroshi, 1954

Table 1 --Continued

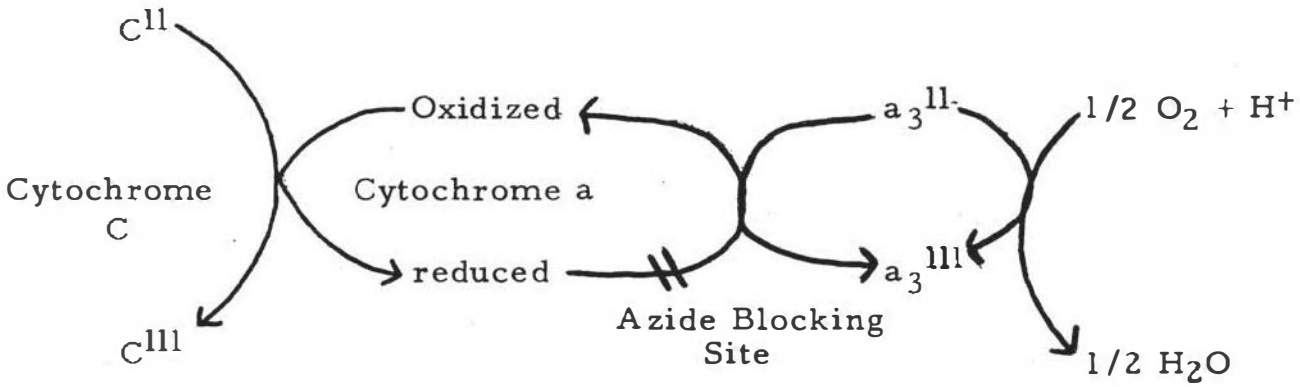
Tissue type or Organism	Author(s)
Inhibition of photosynthesis and/or algae (continued)	
Si uptake in Navicula pelliculosa	Lewin, 1955
Chlamydomonas reinhardtii	Stavis, 1974
Chlamydonomas reinhardtii	Stavis & Hischberg, 1973
Chlorella	Tamya et al., 1958
Chlorella	Wintermans, 1953
Chlorella	Yuan & Daniels, 1956
Inhibition of N_2 Fixation	
Clostridium pasteurianum	Hughes & Welch, 1970
Clostridium pasteurianum	Lockshin & Burris, 1965
Clostridium pasteurianum	Schollhor & Burris, 1967
As a Selective Medium Component	
Streptococcus	Efthymiou & Joseph, 1974
Streptococcus	Mallmann et al., 1937, 1941, 1950
Streptococcus	Snyder & Lichistein, 1940
Miscellaneous Inhibitory Aspects of Azide	
glucose permease in Escherichia coli	Gachelin, 1972
calcium transport in corn	Hodges & Elzam, 1967
soil microflora	Katznelson & Stevenson, 1956
alanine uptake in soybean roots	King & Dleniak, 1973
inhibition of organic acid breakdown in Kalanchoe daigremontiana	Somers, 1951
N_3 distruction by horseradish catalases	Werle, Schievelbein, & Georgopoulos, 1954

TABLE 1--Continued

Medicine*	
auto oxidation of hemglobin	Wallace, 1975
azide-metehemoglobin complexes	Yagisawa, 1974

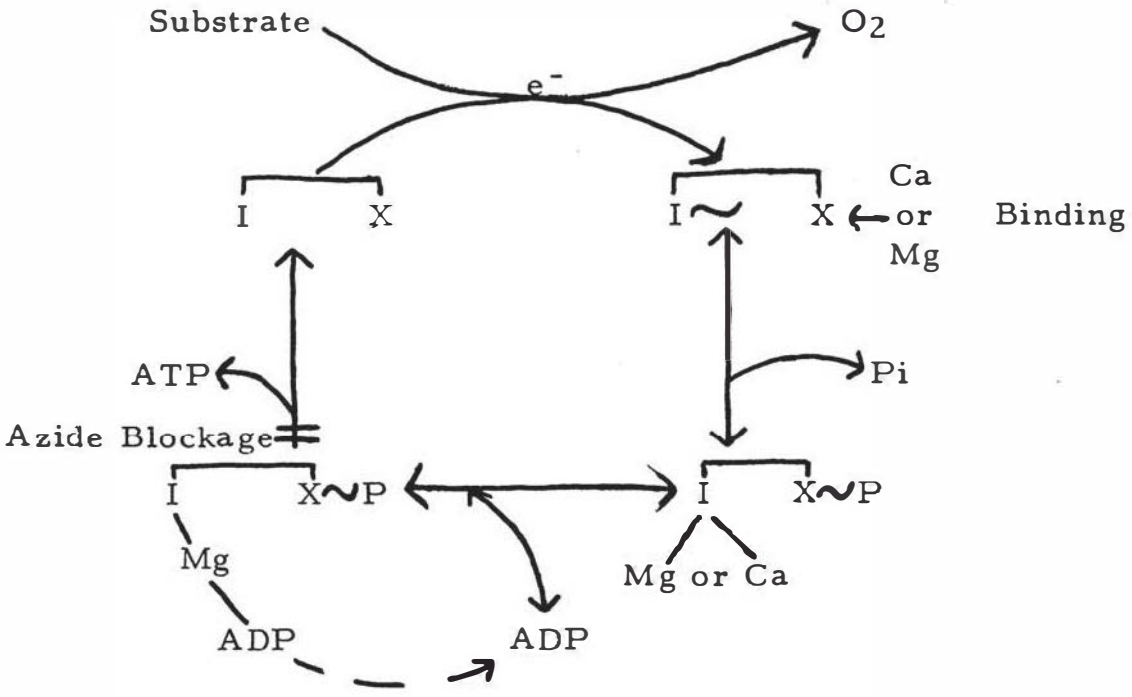
*See Cumulated Index Medicus
U.S. Dept. of Health, Education, and Welfare
Public Health Service
National Institute of Health

for more uses of azide in medicine.



A.

Reported site of Azide Inhibition in Cytochrome System (Yonetani, 1965)



B.

Suggested Site of Azide Inhibition on High Energy Intermediates (Hodges, 1967)

Fig. 1. --Two Mechanisms of Azide Inhibition

much controversy exists as to how azide inhibits formation of ATP from ADP. That azide does inhibit the formation of ATP from ADP, wherever ATP is formed in the cell, is clear from the literature (Table 1), but no one has as yet shown the intermediate steps in the reaction. At present, most physiologists hold that some type of carrier system intimately associated with an electron transport system is necessary for aerobic ATP formation (Hodges, 1972). This type of carrier system and one suggested site of azide inhibition are diagrammed in Figure 1. Inasmuch as there exists no universal agreement with this proposed mechanism, the reader is referred to Table 1 under the heading: "Inhibition of ATP Formation," for other possible mechanisms. Azide concentrations as low as 8×10^{-6} M have been reported by Hodges (1967) to affect ATP formation from ADP.

Even though azide affects living systems, no known living system is able to detoxify azide (Bradbury et al., 1957). Its decomposition appears to be strictly a chemical process accelerated by medium to high acidity, (i.e., pH 0-5), and elevated temperatures and which involves the oxidation of hydrazoic acid (HN_3), formed by azide with organic acids, to form azides of these acids which then decompose by the Curtius rearrangement (see Figure 2). Nitrogen and carbon dioxide gases evolve from these reactions. Azide is also known to readily decompose, via the Curtius rearrangement, in the presence of ultraviolet light (Siderias and Argyrakism, 1974). Thus,

$$RCOOH + HN_3 \longrightarrow RCON_3 + HOH$$

$$RCON_3 \longrightarrow RNCO + N_2$$

$$2RNCO + H_2O \longrightarrow RNCONHR + CO_2$$

Fig 2.--Azide detoxification via the Curtius rearrangement. (Bradbury et al., 1957)

it appears likely that azide, maintained under appropriate environmental conditions, is a reasonably stable compound. With the above considerations in mind, inhibition of organisms grown in azide media over reasonable periods of time (i.e., several weeks) is possible provided (1) that ultraviolet light is not used and (2) that no organic acids are involved in the media. However, there exists the possibility of the production of organic acids by organisms inoculated into and capable of growth in media containing azide. This possibility will be considered further in a later section.

By using the axenic cultures of Chlamydomonas and Carteria, and by growing them on media containing varying sodium azide concentrations, this study will attempt to explore the physiological basis of the effects of sodium azide on various Chlamydomonas and Carteria species. The investigation will concern itself primarily with the possible explanation of growth in azide, if any such growth should occur, and in addition, the utilization of azide as a selective medium component for those species in question.

MATERIALS AND METHODS

The axenic cultures used in this investigation and their sources are listed in Table2 . Cultures were grown in liquid Bold's Basal Medium (BBM; Bold, 1949; Bischoff and Bold, 1963; Cain, 1963; Smith and Bold, 1966), modified by the addition of vitamins B_{12} and thiamine (Smith and Bold, 1966), at the following concentrations of sodium azide; 0%, 10^{-2}%(3mM), 10^{-3}%(0.3mM), 10^{-4}%(0.03mM), 10^{-5}%(0.003mM).

The media were dispersed to culture vessels by means of an automatic pipetting machine (Filamatic Model AB) to insure even, relatively uniform volumes from vessel to vessel in each experiment. Two types of culture vessels were employed, 125-ml Pyrex Erlenmeyer flasks (each containing 40 ml of medium) and 16x150-mm rimless Pyrex test tubes (each containing 10 ml of medium).

Early experimental comparisons between flasks and test tubes showed no significant variation in measured parameters; hence, flasks were eliminated in favor of the convenience of cultivation in test tubes. All vessels were either plugged with non-absorbent cotton or capped with plastic closures prior to sterilization. All media were sterilized by autoclaving at 15 psi (121C) for 15-20 minutes.

TABLE 2

Classification of Organisms Studied
and Their Sources

Chlorophyta
Volvocales
Chlamydomonaceae

	CCIU[a]
Chlamydomonas	
C. eugametos (male)	9
C. eugametos (female)	10
C. reinhardtii (+)	89
C. reinhardtii (-)	90
C. chlamydogama (16-2)	102
C. chlamydogama (16-1)	103
C. actinochloris	965
C. radiata	966
Carteria	
C. crucifera	432
C. eugametos	233
Carteria species	2

[a]Culture Collection number, Indiana University

Aseptic conditions were strictly observed during all inoculations. The inoculations, performed in triplicate throughout, were accomplished initially by means of sterile, cotton-plugged, disposable pipettes equipped with a standard 1-ml rubber bulb used to deliver two drops of a uniform algal suspension to each tube. An alternate method of inoculation, used in later experiments, employed one nichrome wire loop (ca. 3mm diameter) of algal suspension delivered to each tube. The axenic condition of all inocula and experimental cultures were verified by inoculation into half-strength m-Plate Count Broth (Difco). These cultures were initially incubated at 37C for at least 48 hours and subsequently retained at ambient temperatures for periods of up to two weeks to insure adequate incubation for bacterial contamination. Such broth cultures were periodically observed macroscopically for growth and microscopically by examination of smears of culture media and cells stained by Gram's procedure or with crystal violet.

The incubation period for all experiments was 7 weeks, under controlled environmental conditions, henceforth designated as "standard conditions," i.e., a temperature of 22-25C with incident light of 400-500 foot candles (ca 4000-5400 lux) provided by 40-watt Sylvania cool white fluorescent tubes on a 16-hour light, 8-hour dark diurnal cycle. Cursory visual examination for growth at 3 weeks was followed by detailed examination for growth, pH changes, and morphology of each culture at 7 weeks.

Growth was measured either visually with reference to a designated control (qualitative determination) or spectrophotometrically using a Coleman spectrophotometer (quantitative determination). In those experiments where growth was estimated visually the following method of analysis was employed: growth in azide concentrations was compared with that in control media (0.0mM NaN_3) by arbitrary assignment of the number 3 to control tubes; a growth reading of greater than 1 indicates tolerance to azide. All cultures with visual growth evident at 7 weeks were assessed microscopically for any significant changes in size, shape, pyrenoid and chloroplast condition, motility, and general condition of the cells. A Coleman Metrion IV pH meter was used to determine pH changes.

RESULTS AND DISCUSSION

This study represents, to the author's knowledge, the first attempt to demonstrate that selectivity of azide as utilized in bacteriology can be applied in phycology. From the data in Table 3 it is evident that most species studied were capable of at least limited growth in the lowest azide concentration, i.e., 0.003 mM. Both strains of Chlamydomonas reinhardtii (+ and -) showed a significantly high tolerance to increasing azide concentrations (i.e., growth in 0.3 mM NaN_3 was as good as that in 0.0 mM NaN_3). This tolerance suggests the use of azide as a component in selective media for this organism and possibly other azide-tolerant algae. C. reinhardtii grown in 0.3 mM NaN_3 evidenced no morphological differences from those grown in control media. This morphological normalcy was also true of all species which grew in the presence of azide. Because of the unique morphological and physiological tolerance of C. reinhardtii to high azide concentrations, experiments were conducted to determine, if possible, the precise physiological basis of this tolerance.

Several explanations of azide tolerance may be postulated: (1) azide is utilized as a nitrogen source; (2) C. reinhardtii contains little or no cytochrome a, the commonly accepted site of azide inhibi-

TABLE 3

Visual Estimates of Growth after 7 Weeks in Azide-supplemented Bold's Basal Medium[c]

Organism	Azide Concentration (mM/l)				
	0	.003	.03	0.3	3.0
Chlamydomonas eugametos ♂	3[a]	t	t	0	0
	3[b]	2	2	0	0
C. eugametos ♀	3	2	2	t	0
	3	3	1	t	0
C. reinhardtii +	3	3	3	3	0
	3	3	3	3	0
C. reinhardtii -	3	3	3	3	0
	3	3	3	3	0
C. chlamydogama (16-2)	3	t	t	0	0
	3	0	0	0	0
C. chlamydogama (16-1)	3	t	0	0	0
	3	t	0	0	0
C. actinochloris	3	2	1	t	0
	3	2	t	0	0
C. radiata	3	3	t	t	0
	3	4	t	0	0
Carteria cruciferia	3	3	3	0	0
	3	t	t	0	0
C. eugametos	3	3	t	0	0
	3	3	t	0	0
Carteria species	3	0	0	0	0
	3	3	t	0	0

[a] Exp. 1
[b] Exp. 2
[c] 3 = maximum growth (control)
2 = growth
1 = growth
t = trace growth
0 = no growth

tion; (3) C. reinhardtii has cytochrome systems differing significantly from that of most organisms so that azide does not affect its respiratory or photosynthetic processes; (4) C. reinhardtii in some way circumvents azide inhibition of formation of ATP from ADP; (5) there is a permeability barrier which does not allow HN_3 to enter the cell (Steppani, 1949; Sideris and Argyrakism, 1974); (6) C. reinhardtii excretes extracellular products, most notably organic acids, which may detoxify azide via the Curtius rearrangement (Figure 2); and (7) decomposition of azide over extended periods of time may occur (Mallmann, Botwright, Churchill, 1937; Decker and Tanner, 1972). In this study, efforts were, therefore, undertaken to determine which, if any, of the above cited explanations is/are applicable to azide tolerance as exhibited by C. reinhardtii.

The first of the above possibilities, that azide might act as a source of nitrogen for C. reinhardtii, was investigated by growing C. reinhardtii with NaN_3 as the sole nitrogen source in varying concentrations, i.e., 0.003mM, 0.03mM, 0.3mM, and 3.0mM azide. The results showed that azide is not used as a nitrogen source by C. reinhardtii. Experiments such as this with other algal species listed in Table 2 similarly showed that azide cannot serve as a source of nitrogen for these organisms.

The second possibility, i.e., that C. reinhardtii lacks significant amounts of cytochrome a, is not easily approached experimentally

due to the chemical complexity in the isolation and identification of this cytochrome. Chance and Sager (1957) and others (Smillie and Levine, 1963; Givan and Levine, 1967; Levine and Smillie, 1967; Chua and Levine, 1969) have created mutants of C. reinhardtii lacking various cytochromes for studies of photosystems I and II electron transfer reactions; one of these mutants lacked cytochrome a. Since all known wild-type C. reinhardtii species do contain detectable amounts of cytochrome a, it appears unlikely, although not impossible, that the strains used in these experiments performed in triplicate would show such consistency in replication if a mutant was present since this would imply consistent mutation of wild-type C. reinhardtii after inoculation. To imply that after inoculation three separate mutations of C. reinhardtii occurred and, that all occurred at the same time and showed the same amount of growth is highly unlikely. Even though the possibility cannot be completely eliminated from consideration, it appears highly improbable that in all experiments conducted, two mutant strains of C. reinhardtii lacking cytochrome a were isolated.

The third possibility, that C. reinhardtii has cytochrome systems differing significantly from that of most organism so that azide does not affect its respiratory or photosynthetic processes, appears unlikely since in the literature dealing with C. reinhardtii and the electron transport chains of photosynthesis there is no mention of such a radically different means of electron transport (Smillie and

Levine, 1963; Givan and Levine, 1967; Levine and Smillie, 1967; Chua and Levine, 1969). Since the C. reinhardtii cytochrome system appears to be the same as that of most other organisms, one must assume that this possible explanation of azide tolerance has no credible support.

The fourth possibility, that C. reinhardtii circumvents azide inhibition of the formation of ATP from ADP is again not substantiated by the literature (Smillie and Levine, 1963; Givan and Levine, 1967; Levine and Smillie, 1967; Chau and Levine, 1969), nor our present knowledge of C. reinhardtii. Such a circumvention would imply an entirely new means and/or mechanism of energy formation. Since ATP formation is often intimately associated with the respiratory cytochrome system and the cytochrome system in C. reinhardtii is considered to be similar to that of all organisms, it is highly unlikely that C. reinhardtii circumvents azide inhibition of ATP formation regardless of where ATP formation is taking place, i.e., Krebs' cycle, glycolysis, cytochrome system(s).

The fifth possibility, i.e., that there is a permeability barrier which does not allow HN_3 to enter cells of C. reinhardtii, appears much more probable than any of the foregoing. Phototactic studies by Stavis (1974) on stationary or log phase cultures of C. reinhardtii showed no apparent affect of azide (0.350 mM) on motility (observed for thirty minutes), respiration, or ATP production. Azide did affect some unknown intermediate in the phototactic response mechanisms, but no explanation of azide tolerance is given. Stavis found no change

in measurable O_2 uptake with azide concentrations up to 0.35 mM and no change in ATP production with the firefly luciferin/luciferase assay at the aforementioned azide concentrations. These results imply the existence of a possible permeability barrier in which hydrazoic acid (HN_3) is either not entering the cell or, if it is, not entering in significant quantities to inhibit motility, respiration and ATP production. This implication is justified by the lack of apparent effect of azide on respiration and ATP production shown by Stavis.

The existence of such a permeability barrier is also indirectly implied by studies on azide inhibition in Chlorella (Yuan, 1956; Decker and Tanner, 1972). Yuan showed inhibitory effects of 0.016 to 4.5 mM concentrations of azide on Chlorella sp. Azide inhibited photosynthesis at all concentrations, stimulated respiration up to 1.96 mM and inhibited respiration (up to 21%) at 3.5 to 4.6 mM. Decker and Tanner showed that the steady-state influx of hexokinase in Chlorella vulgaris was inhibited by 10.0 mM azide. Influx of hexokinase in Chlorella vulgaris is controlled by an active carrier system needing energy in the form of ATP to function. Thus the inhibitory effects of azide on respiration, photosynthesis and ATP production in Chlorella probably implies a permeability barrier present in C. reinhardtii, assuming these two organisms to be physiologically similar.

If permeability is a factor, there should be a critical concentration of azide which inhibits growth. This critical concentration

appears to be between 0.3 and 3.0 mM azide (Table 3). It is generally assumed that the plasma membrane of young cells is more permeable than that of older cells (Stadelmann, 1962). If cell age is a factor, one would predict that older inocula of C. reinhardtii would be unaffected by azide concentrations of less than 3.0 mM. Regardless of this fact, no explanation of how newly-dividing cells with greater permeability could tolerate azide already present in the medium exists, unless young cells also show a permeability barrier. Young cells of C. reinhardtii appear to have this permeability barrier as evidenced by the ability of C. reinhardtii to show significant growth and reproduction in 0.3 mM azide.

The hypothesis that there is a permeability barrier which does not allow HN_3 to enter the cell was further investigated by an experiment designed to determine the effects of pH on azide toxicity since a high pH, i.e., pH 8 or above, may significantly alter the concentration of HN_3 present in the medium and therefore the amount entering the cell. Media were adjusted to pH values of 6.0, 7.0, 8.0, and 9.0 at various azide concentrations. "Tris" buffer was used in the alkaline media in order to maintain stable pH levels. As a control, i.e., to insure that azide inhibition was occurring and that no azide deterioration was taking place, an azide-sensitive organism, Chlamydomonas chlamydogama, (83) was used. This organism (see Table 3) showed only trace growth in 0.003 mM azide and no growth at higher concen-

trations. Therefore, any growth of C. chlamydogama at azide concentrations higher than 0.003mM would indicate that azide toxicity was not occurring. The results of this experiment are presented in Table 4.

Several observations can be made from these data. At pH 6, in the highest azide concentration (0.3mM), growth of C. reinhardtii is significantly less than in the control (0.0mM). This result can be attributed to the fact that more HN_3 is present at acidic pH levels and thus more of it would enter the cell and inhibit growth. Likewise, at pH 9, less HN_3 should be present and, therefore, less inhibition would be expected and better growth should occur. The data, with isolated exceptions, tend to support these observations for C. reinhardtii. The results for C. chlamydogama, the azide-sensitive organism, serve as a good example of how pH affects the amount of HN_3 and, therefore, the degree of inhibition of growth; C. chlamydogama showed good growth only at pH 9 at 0.003 and 0.03mM azide concentrations. These data then further substantiate the permeability barrier belief based on the concept that the greater the concentration of HN_3 in the media (i.e., lower pH, 0-6 more HN_3 present), the more likely HN_3 is to enter the cell and cause inhibition of growth.

Table 4 also shows two other interesting observations. There is a difference between measurement of growth visually and photometrically. Although visual growth measurements show qualitative trends quite well, quantitative determination by absorbance measurements is

TABLE 4

Effect of pH on Azide Toxicity[a]

Initial pH	Range Final pH Azide Concentration (mM)				Visual[c] Azide Concentration (mM)				Absorbance[b] Azide Concentration (mM)			
	0.0	0.003	0.03	0.3	0.0	0.003	0.03	0.3	0.0	0.003	0.03	0.3
C. reinhardtii (+)												
6	6.7-6.9	6.6-6.7	6.8-7.3	6.4-6.5	3	3	3	1	.456	.367	.523	.276
					3	3	3	1	.347	.347	.310	.187
					0	4	3	0	.001	.585	.244	.051
7	6.8-7.2	7.0-7.1	6.7-7.0	6.7-7.0	3	3	1	3	.796	1.000	.284	.699
					3	4	1	2	.678	1.000	.168	.495
					3	3	1	1	.444	.523	.155	.143
8	7.2-7.4	7.1	7.0-7.3	7.4-7.5	3	5	3	6	.108	.208	.208	1.046
					3	3	3	4	.125	.097	.168	.284
					3	3	3	5	.056	.155	.155	.638
9	7.6-7.8	7.7-7.9	7.8-8.1	7.9	3	5	4	3	.569	1.097	.638	.721
					3	6	5	4	.194	1.155	.301	.678
					3	6	4	4	.194	1.222	.268	.678
C. reinhardtii (-)												
6	6.7-6.8	6.8-6.9	6.6-6.9	6.0-6.5	3	2	2	0	.357	.229	.367	.009
					3	2	2	0	.310	.367	.337	.013
					3	3	2	0	.337	.569	.409	.004
7	6.7-6.9	6.7-6.9	6.3-6.5	6.2-6.6	t	1	1	1	.041	.051	.071	.155
					t	1	1	0	.046	.143	.056	.018
					3	5	3	6	.076	.392	.071	.699
8	7.4-7.5	7.0-7.4	7.0-7.4	7.0	3	2	3	3	.108	.456	.319	.377
					3	5	3	3	.155	.678	.047	.131
					3	3	5	0	.538	.569	.886	.027
9	7.8-8.1	7.8-8.1	7.7-8.1	7.8-7.9	3	5	4	4	.301	1.000	.432	.854
					3	5	4	4	.252	1.097	.174	1.000
					3	5	2	4	.252	1.097	.187	.854

TABLE 4--Continued

Initial pH	Range Final pH Azide Concentration (mM)				Visual[c] Azide Concentration (mM)				Absorbance[b] Azide Concentration (mM)			
	0.0	0.003	0.03	0.3	0.0	0.003	0.03	0.3	0.0	0.003	0.03	0.3
C. chlamydogama (16-2)												
9	7.6-7.7	7.7-7.8	7.6-7.8		3	5	3		.602	.721	.252	
					3	4	3		.292	.638	.237	
					3	6	2		.222	.770	.168	

[a]Incubation period of 7 weeks

[b]650 nm

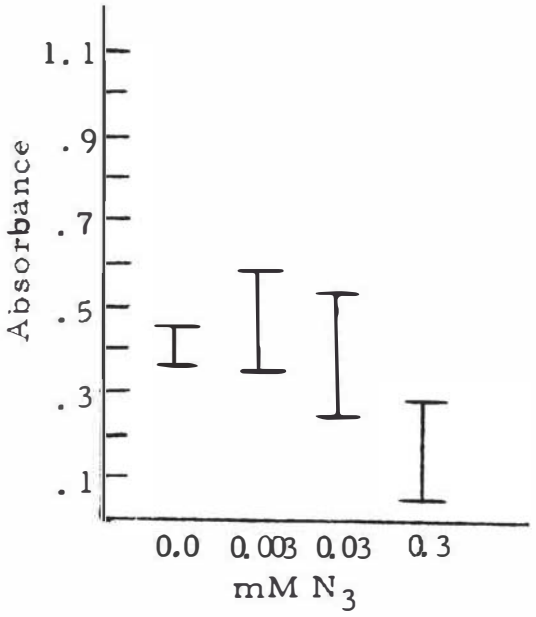
[c]Control media - arbitrary 3

necessary to insure accuracy and precision. Since this research was not primarily concerned with quantitative growth measurements, visual estimates of growth were considered adequate to show effects of azide on the organisms studied. Figure 3 shows an example of how much growth can vary in these organisms, incubated under standard conditions.

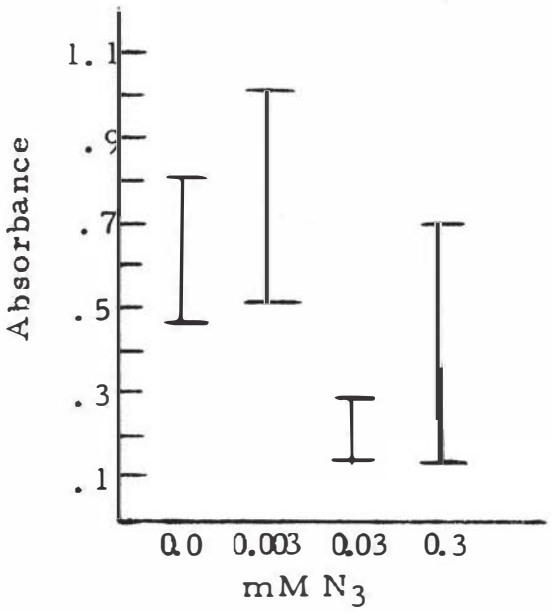
The second observation, represented in Figure 4, is the fact that the initial pH values shifted and that variations in final pH values for an organism at the same initial pH was not uncommon. The pH shift, in all cases, was towards neutrality. This shift suggests the possibility of production of extracellular products and/or preferential absorption of one or more nutrients with a concomitant pH change. These extracellular products may/may not shift pH levels but do possibly, if acidic, detoxify azide via the Curtius rearrangement.

To explore the possibility that extracellular products were being produced by C. reinhardtii, that detoxified azide, two experiments using biological assay procedures were conducted.

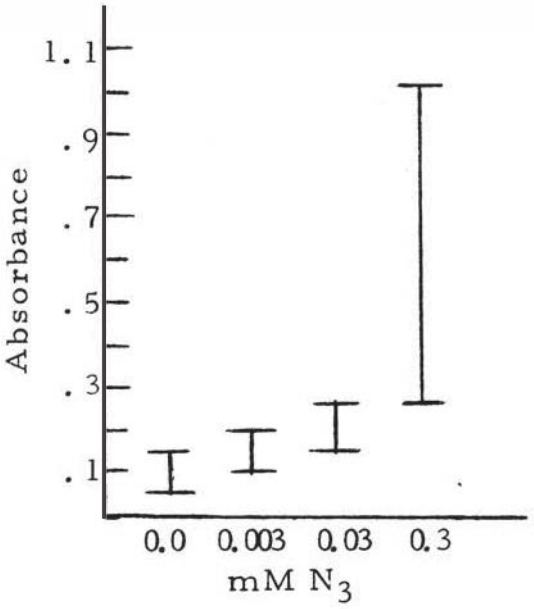
In the first experiment, parabiotic tubes (U-tubes), each side of which was separated by a 0.20 uM Metricel filter (GA-8) were employed. The tubes were inoculated on one side with the azide-tolerant C. reinhardtii; the sensitive species, C. chlamydogama, was then inoculated into the opposite side. It was assumed that sufficient time for growth of C. reinhardtii was necessary before sufficient extracellular



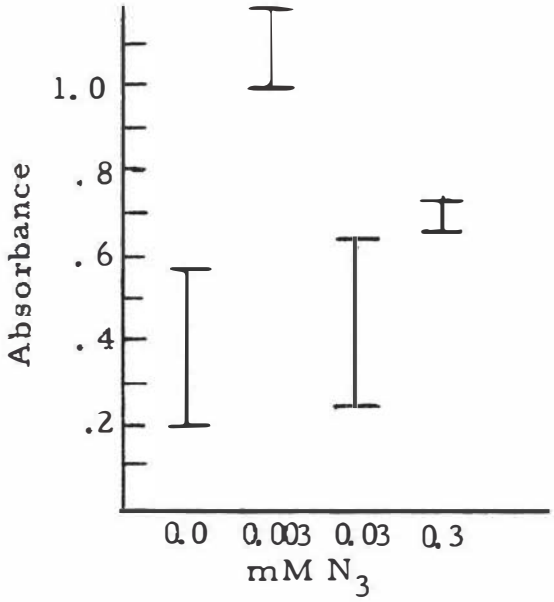
pH 6



pH 7



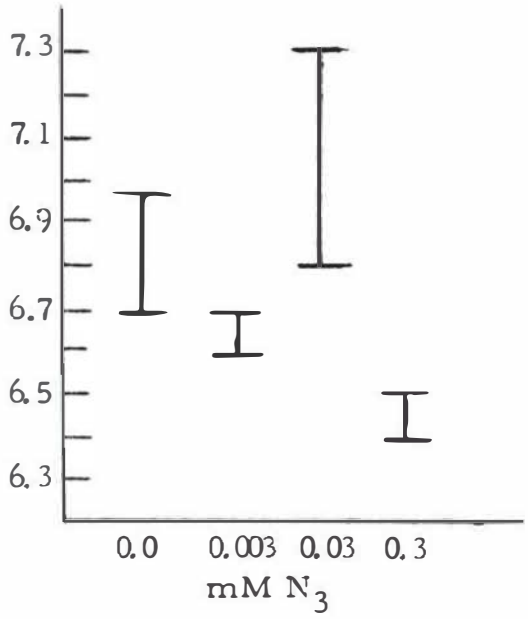
pH 8



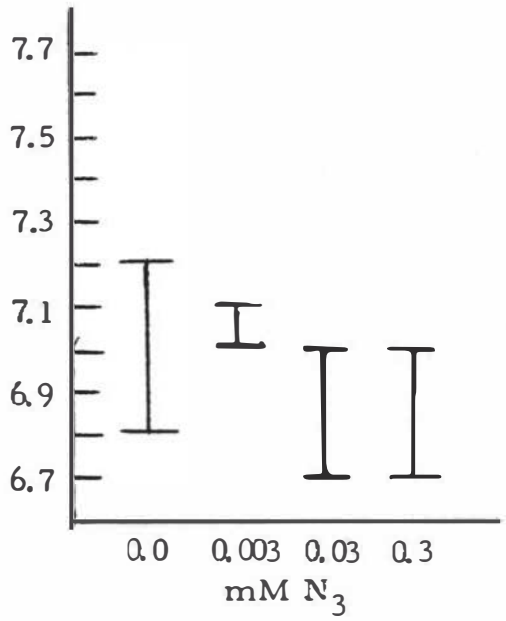
pH 9

Fig.3 . --Representative Growth Ranges Measured Using Absorbance Recordings[a] for C. reinhardtii(+)[b] in Varying Azide Concentrations.

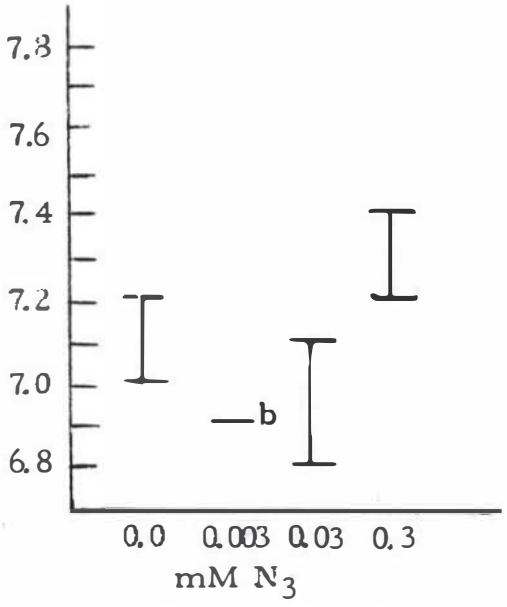
a = 650 nm b = 7 weeks, growth



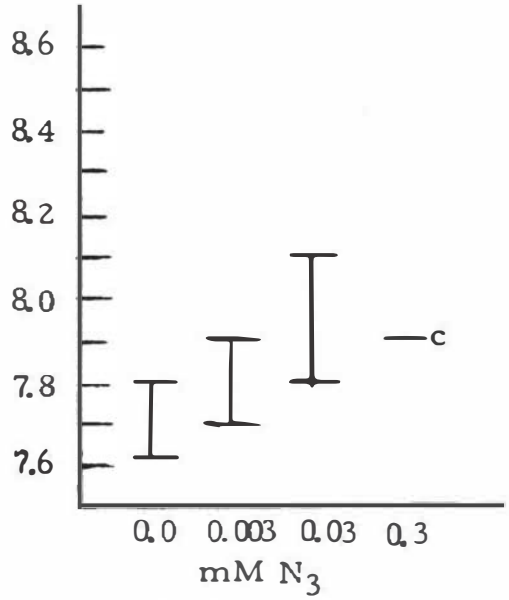
Initial pH 6.0



Initial pH 7.0



Initial pH 8.0



Initial pH 9.0

Fig.4 .--Representative Final pH Ranges for C. reinhardtii (+) in Varying Azide Concentrations[a]

a = After 7 weeks, growth b, c = All readings same

production, if any, could take place to detoxify the azide. Accordingly, the C. chlamydogama sides were re-inoculated at one-week intervals and observed for visual growth on a day-to-day basis. If, after the previous week's inoculation, no growth occurred, sufficient azide must have been present to inhibit growth of C. chlamydogama, and reinoculation of C. chlamydogama would be necessary. Visible growth of C. chlamydogama was considered as evidence for the excretion of some extracellular product by C. reinhardtii which, in turn, detoxified the azide. Appropriate controls, i.e., 0.0mM azide, were also employed to rule out the possibility that C. reinhardtii changed the medium in some other manner. Only 0.3mM azide was used since this was the concentration experimentally determined (Table 3) to be the highest allowing growth of the resistant, but not of the sensitive, species.

The second experiment conducted to determine biological degradation of azide by extracellular products produced by C. reinhardtii also employed biological assay. In this experiment, media in which C. reinhardtii had been grown for 7 weeks (0.3mM azide) were suction-filtered using a 0.20uM Metricel filter (GA-8). The filtered media were then inoculated with the azide-sensitive C. chlamydogama and incubated for 7 weeks. These cultures were compared with fresh media (also 0.3mM azide) inoculated with C. chlamydogama, incubated also for 7 weeks. Again, growth of C. chlamydogama in the filtered 0.3mM azide media was considered to indicate that extracellular products of C. reinhardtii possibly detoxified the azide.

Tables 5 and 6 show the results of these experiments investigating extracellular production. The results show that C. reinhardtii was either excreting a substance which detoxified azide or was neutralizing azide once it entered the cell. If azide were entering the cell there is no reason to suspect that azide would not inhibit cytochrome a and ATP formation from ADP, therefore, the possibility that azide is detoxified after entering the cell before azide inhibition can occur appears improbable. The ability of various freshwater Chlamydomonas species to produce extracellular organic matter (10 to 45% into culture media) has been known for some time (Allen, 1956). This fact, along with the lack of evidence for biological degradation of azide (Bradbury et al., 1957) and with the results of the present investigation, suggests that C. reinhardtii may possibly excrete extracellular organic matter which detoxifies azide via the Curtius rearrangement.

From the above evidence it appears that a combination of two possible means of azide tolerance may be exhibited by C. reinhardtii: (1) first a permeability barrier may exist which does not allow sufficient HN_3 to enter the cell to inhibit, sequentially followed by (2) "detoxification" of azide via the Curtius rearrangement, which occurs after sufficient cell numbers are present and excretion of large quantities of extracellular products which are probably largely organic.

Ultrastructural studies of C. reinhardtii (Sager and Palade, 1957; Lembi and Lang, 1965) lend further support to the above two pos-

TABLE 5

Parabiotic Demonstration of Azide Detoxification by Chlamydomonas reinhardtii[a]

	0.0mM N_3		0.3mM N_3	
	C. chlamydogama	C. reinhardtii	C. chlamydogama	C. reinhardtii
1 week	+	+	-	+
2 weeks	+	+	-	+
3 weeks	+	+	-	+
4 weeks	+	+	+[b]	+

[a]See text for discussion

[b]Detoxification

TABLE 6

Detoxification of Azide Media by Chlamydomonas reinhardtii after 3 Weeks' Incubation in Azide and C. reinhardtii Filtrate Media[a]

pH	Azide Concentration 0.003mM	0.03mM	0.3mM	C. reinhardtii Filtrate[b]
6	-	-	-	+
7	-	-	-	+
8	-	-	-	+
9	t	t	-	+

[a]See text for discussion

[b]Original concentration of NaN_3 0.3mM/l

sibilities. These studies show a layer of galacturonic acid in the cell wall which may presumably aid in detoxification or prevent penetration of azide, as well as a copious mucilaginous excretion consisting of protein, cellulose fibers (occasionally) and galacturonic acid, some of which may affect azide. It should also be noted that Carteria eugametos, Carteria crucifera, and Carteria species show cell wall ultrastructure differences when compared with C. reinhardtii and C. eugametos. These differences encourage speculation that mucilage composition may be important as a permeability barrier (Lembi and Lang, 1965).

The effects of both time and pH on azide toxicity were also investigated to eliminate these factors as a possible explanation of algal growth in azide-supplemented media. Various pH levels and azide concentrations were prepared as previously described. Media were allowed to stand uninoculated for 16 weeks under standard conditions. After the 16-week period, bioassay procedures were again employed by inoculating C. chlamydogama into the media. Growth was observed over a 7-week period. Visual growth of C. chlamydogama indicated azide detoxification. Fresh media adjusted to the same pH levels and azide concentrations were inoculated with C. chlamydogama and observed for visual growth over the same 7-week period as controls.

The results (Table 7) show that time has no appreciable effect on azide detoxification at pH levels of 7 and 8 but that some azide detoxification does occur at pH levels of 6 and 9. Although acidic condi-

TABLE 7

Effect of Culture Medium Age on Growth of *Chlamydomonas chlamydogama* in Varying Concentrations of Azide and Varying pH Values[a]

pH	0.0	0.003mM	0.03mM	0.3mM
6	_[b]	-	-	-
7	-	-	-	-
8	t	t	-	-
9	+	+	+	-

[a] Media were prepared and stored under standard conditions for 16 weeks prior to inoculation

[b] Growth: + = Growth; t= trace; - = no growth

tions (pH<6) have been shown to lead to rapid azide detoxification via the Curtius rearrangement (Bradbury et al., 1957) no reasonable explanation of detoxification at highly basic pH levels is found in the literature. Since the incubation period of all experiments was 7 weeks, there appears to be little probability that azide detoxification took place over the incubation period of these experiments.

GENERAL CONCLUSIONS

From the above experimentation and discussion it appears possible that C. reinhardtii can grow in azide concentrations up to 0. 3mM because of a permeability barrier. After sufficient cell numbers have developed, azide is then detoxified by the excretion of extracellular organic matter via the Curtius rearrangement. Azide is not capable of being utilized as a nitrogen source nor is azide degraded significantly over the 7 week period under the standard conditions under which these experiments were conducted. The pH of the medium is important since (1) cultures in acidic media (pH 6) are more greatly inhibited and (2) there occurs a more rapid degradation of azide (via the Curtius rearrangement in uninoculated acidic media.

SUMMARY AND CONCLUDING REMARKS

A comparative study of 8 species of taxonomically similar axenic cultures of freshwater algae (5 species of the genus Chlamydomonas and 3 species of the genus Carteria) was undertaken in order (1) to determine their ability to grow in an inorganic medium supplemented with vitamins B_{12} and thiamine, and to which was added 0.3, 0.03, and 0.003mM sodium azide, (2) to explain growth in azide, if such growth should occur, and (3) to show the possible utilization of azide as a selective agent in media for those organisms in question.

Only Chlamydomonas reinhardtii strains showed significant growth in media containing this highest concentration (i.e., 0.3mM) of azide. C. eugametos (male and female), C. chlamydogama strains, C. actinochloris, and C. radiata all showed limited growth in 0.003-0.03mM azide, as did Carteria crucifera, Carteria eugametos, and a Carteria sp. No morphological changes of organisms grown in media containing azide were observed. Azide was not utilized as a sole nitrogen source by any of the organisms capable of growth in varying azide concentrations. The effects of pH and time on azide toxicity were discussed, as were the importance of permeability and extracellular products as factors involved in azide toxicity.

Two possibilities exist to explain growth of the C. reinhardtii strains in media containing 0. 3mM azide: (1) there exists the possibility of a permeability barrier in C. reinhardtii which does not allow azide to penetrate the cell and (2) the strong possibility exists that C. reinhardtii may excrete extracellular products (organic acids) which may detoxify azide via the Curtius rearrangement.

It would have been advantageous if an experiment were run to show beyond a doubt that a permeability barrier is the precise means by which C. reinhardtii tolerates azide. Such an experiment is possible by measuring the rates of respiration and photosynthesis with a respirometer and the use of various substances such as low concentrations of alcohol or detergents which typically increase membrane permeability. By running the appropriate controls, varying the azide concentrations, and increasing the permeability of the cell using alcohols or detergents, measurements of respiration and photosynthesis could be taken to show whether azide is or is not entering the cells of C. reinhardtii and whether it is or is not affecting the photosynthetic and/or respiratory processes.

Alternately, observations of C. reinhardtii motility as first suggested by Stavis (1974), employing the aforementioned techniques, may also show whether azide is entering the cells since motility necessitates the consumption of energy (ATP).

This investigation may be useful to ecological studies for the

detection of known as well as yet unidentified azide-tolerant algal species from soil and water. Certain Chlorosarcinalean and Chlorococcalean algae have been isolated by the author from soil samples using media supplemented with azide at various concentrations. The problems associated with the identification of such organisms involves tedious morphological and life cycle observations beyond the scope of this study.

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