Nor-Carotenoids as the Major Volatile Excretion Products of *Cyanidium*

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Cyanidium caldarium excretes a series of nor-carotenoids. A 110 litre culture was used to isolate and determine the structure of 5 compounds: methylheptone, geranylacetone, β-ionone, dihydrotrimethylnaphthalene, butenylidenetrimethylcyclohexene. An algal bioassay was used to determine their effectivness on the growth of photoautotrophic organisms. The growth of *Anabaena*, *Synechococcus*, *Nannochloris* and *Cyanidium* was found to be inhibited at concentrations between 10 to 50 ppm.

During the growth of algal blooms, natural fresh waters frequently acquire odours characteristic of the dominant algal species. The odours have been described as being fishy, musty and septic or sweet and of violets and geraniums [1, 2]. The chemical nature of a very few of these odours has been determined probably due to their very low concentrations in natural waters and the resulting analytical problems concerned. One of the first examinations of an algal odour was performed with the subaerial *Trentepohlia iolithus* which produced in the natural habit the so-called "Veilchenstein". The typically violet odour was found to be produced by β-ionone [3]. Geosmin has been shown for many years to be the causative agent for musty-earthy aromas found in association with Cyanobacteria and Actinomycetes [4, 5] and its structure has been determined as a *trans*-1,10-dimethyl-*trans*-9-decalol [6]. The studies of Tabachek and Yurkowski [7] have demonstrated its wide distribution in Cyanobacteria and in a few cases 2-methylisoborneol has also been found. An additional group of compounds is represented by aldehydes, extensively studied in *Synura petersenii* and *Cryptomonas ovata* by Collins and Kalnins [8, 9]. This group of compounds was found after steam distillation of algal cultures. Steam distillates of *Chlorella* species have however yielded limonene, myrcene and eucalyptol [10]. Wether these compounds can be liberated under natural conditions from these species is not known. Amines were found to be responsible for the fishy odour of the cultures of a variety of algal species [11]. During investigations on a bloom of *Microcystis wesenbergii* in the Federsee we isolated the tobacco like odorous compound β-cyclocitral [12]. Now we would like to report on the aromatic odour of *Cyanidium caldarium* which is produced by a series of nor-carotenoids previously unknown in microalgae.

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Materials and Methods

Precultivation of the algae

Cyanidium caldarium strain 1355/1 Allen was obtained from the Culture Centre of Algae and Protozoa, Cambridge, *Anabaena variabilis* from Dr. N. G. Carr, Department of Biochemistry, University of Liverpool, *Synechococcus* 6911 from Prof. R. Y. Stanier, Institut Pasteur, Paris, *Nannochloris coccoides* No. 251-1 from the Sammlung von Algenkulturen des Pflanzenphysiologischen Instituts, Göttingen. The bioassay test organisms *Anabaena*, *Synechococcus* and *Nannochloris* were grown in a medium of Jüttner [12] at 27 °C in 300 ml cultivation tubes (see Kuhl and Lorenzen [13]) with 0.2% CO_2 air aeration. *Cyanidium* was cultivated at 30 °C, sparged with 1.2% CO_2 in air using a medium of Allen [14] with minor modifications. Full details of which are as follows: In 1 litre of distilled water, the following salts were added: 1.3 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7\,H_2O$, 0.26 g KH_2PO_4, 74 mg $CaCl_2 \cdot 2\,H_2O$, 3.1 mg H_3BO_3, 2 mg $MnCl_2 \cdot 4\,H_2O$, 290 μg $ZnSO_4 \cdot 7\,H_2O$, 480 μg $Na_2MoO_4 \cdot 2\,H_2O$, 50 μg $CuSO_4 \cdot 5\,H_2O$, 50 μg $CoCl_2 \cdot 6\,H_2O$ and 3.7 mg Na-Fe^{III}-ethylenediamine tetraacetate (Fluka). The pH of the medium was adjusted to 3 with ammonia before autoclaving.

Mass production of Cyanidium caldarium

The mass cultivation of *Cyanidium* was performed in a 110 litre algal pilot plant [15] illuminated with 54 fluorescent tubes (Osram Interna 39/40 w) which produced a light intensity of 6000 lx (3×10^4 erg · $cm^{-2} \cdot s^{-1}$) at the surface of the culture apparatus. The agal cell suspension was circulated at a rate of 25 m/min through the 4 cm i. d. glass tubes. The temperature was held at 30 – 31 °C and the gas flow rate at 360 l/h. A semicontinuous culturing procedure was performed. 95 litre of the algal suspension were harvested at weekly intervals. Details of sterilization, inoculation and harvesting were similar to those previously outlined [16]. The average weekly yield of *Cyanidium* was 750 g wet weight.

Production and concentration of the odorous compounds

The stripping method was used to separate the odorous compounds from the algal suspension. The gases from the algal cultivation plant were precooled to +1 °C by passing a thermostatisized condenser which removed most of its water. The precooled gases were trapped by succession in a 4 cm i. d. U-tube to remove the bulk of the water vapour, then another 1.5 cm i. d. U-tube to remove residual water and finaly three 55 cm high 3 mm i. d. loops half dipped in a dry ice-acetone bath. This arrangement markedly reduced the formation of aerosols. The condensate of the two major traps was removed daily. The loops were washed with a small amount of diethyl ether every 3 days. The ether washings and the condensed water were united and stored. 125 g of water condensate collected within 7 days were extracted 3 times with a total of 70 ml diethyl ether and the ether extract dried over Na_2SO_4. The diethyl ether used was previously distilled over Na and paraffin wax. The residue of 10 ml of which upon evaporation exhibited no background in the gas chromatographic separation at the sensitivity usually used for analysis of the odorous compounds.

Gas chromatography and mass spectrometry

The etheric extract was gently concentrated in a rotary evaporator to 400 µl. 2 µl were injected into a gas chromatograph (model Carlo Erba 180) with an integrator (Supergrator-2/Columbia Scientific Industries) equipped with a 15 m OV-101 WCOT glass capillary column. H_2 was used as the carrier gas (100 ml/min with a split ratio of 1 : 30). The flame ionisation detector and the injection port were maintained at 250 °C. A temperature program from 40 °C to 180 °C with 5 °C/min was chosen. The mass spectra were obtained by a LKB 9000 at a ionizing energy of 70 eV, the same column but helium as carrier gas were used for separation.

Preparative separation and hydrogenation

Preparative separation of the etheric extract into 7 fractions was achieved with a Hewlett Packard 5750 G gas chromatograph with a miniprep. A packed 1.8 m long and 3 mm i. d. glass column (3% OV-17 on Chromosorb W AW DMCS 80 – 100 mesh) was used. The eluted fractions were trapped in glass vials cooled in a dry ice-acetone bath. For hydrogenation the fractions were solved in 20 µl diethyl ether, supplied with a trace of PtO_2 and hydrogenated for 2 h.

Reference substances

β-Ionone was purchased from Fluka/Neu-Ulm/Germany; methylheptenone was a kind gift of the Teranol AG, Lalden/Switzerland and a mixture of *cis*- and *trans*-geranylacetone was obtained from BASF/Ludwigshafen/Germany. 1,2-Dihydro-1,1,6-trimethylnaphthalene was prepared after the method of Karrer and Ochsner [17] from β-ionone. A pure sample was obtained by distillation and preparative gas chromatography. The 6-(but-2-enylidene)-1,5,5-trimethylcyclohex-1-enes were obtained after treatment of β-ionol with $POCl_3$ in pyridine. The reaction mixture of which was separated by preparative gas chromatography [18] on a 9 mm i. d. and 1.8 m glass column filled with 3% OV-17 on Chromosorb W AW DMCS 80 – 100 mesh, using a carrier gas flow of 120 ml/min and a split ratio of 1 : 15. The UV spectra were recorded in diethyl ether on a Cary spectrophotometer. The saturated compounds were synthesized by hydrogenation with H_2/PtO_2 from the unsaturated ones.

Algal bioassay

300 ml Erlenmeyer flasks filled with 100 ml algal suspension were incubated in a controlled environment incubator (model G 27, New Brunswick Sci.

Co.) with shaking at 150 rev/min. During the growth experiments on the Cyanobacteria, an incident light intensity on the cultures of 1400 lx was used, and the incubator was aerated with 0.045% CO_2 in air at a flow rate of 500 ml/min. For that of Cyanidium and Nannochloris a light intensity of 4500 lx and an aeration rate of 500 ml/min (1.5% CO_2 in air) was used. The CO_2-air mixture was controlled by an infra red gas analyser (model 20, Grubb Parsons/ Newcastle upon Tyne, UK). The culture density was determined colorimetrically (model E 1009, Metrohm) twice a day at 550 nm. The nor-carotenoids were added in the bioassays when the cultures had reached $E_{550}^{1.0\,cm} = 0.1$. Quantities of less than 1 μl were diluted with ethanol 1 : 10 (v/v) before addition.

Results

The aroma of *Cyanidium caldarium* can easily be recognized in small scale cultures as well as in mass culture. It resembles the odour, Cyanobacteria and Chlorophyceae often impart to the culture media. With culture sparging the bulk of these odorous compounds were stripped from the pilot plant. Extraction of the culture medium with diethyl ether or sublimation of the algae themselves (400 g wet weight) lead only to minor amounts of these compounds produced. Though a 110 litre mass culture of *Cyanidium* was used to produce the odorous compounds, μg amounts of a single compound were never surpassed, *e. g.* not more than 0.7 μg/d of β-ionone were obtained. Therefore, the identification of these compounds could only be performed using gas chromatography, mass and UV spectrometry, combined with microchemical reactions.

The odour of *Cyanidium* is represented by a mixture of several compounds which can be separated on a WCOT glass capillary column. Fig. 1 shows such a separation of odorous compounds found in the ether extract of the trapped condensate. The mass spectra of peak 1 (*m/e* 43(100%), 41(49%), 69(38%), 55(34%), 108(29%), 31(21%), 58(18%), 111(16%), 71(16%), 39(16%), 68(15%)) and 13 (*m/e* 177(100%), 43(61%), 44(26%), 41 (22%), 159(20%), 93(18%), 91(18%), 149(16%), 135(16%), 178(14%), 192(13%)) were very similar to those reported for 6-methylhept-5-en-2-one (**IV**) and β-ionone (**I**) [19]. Hydrogenation of the ether extract lead to the formation of compounds which allowed the mass spectra

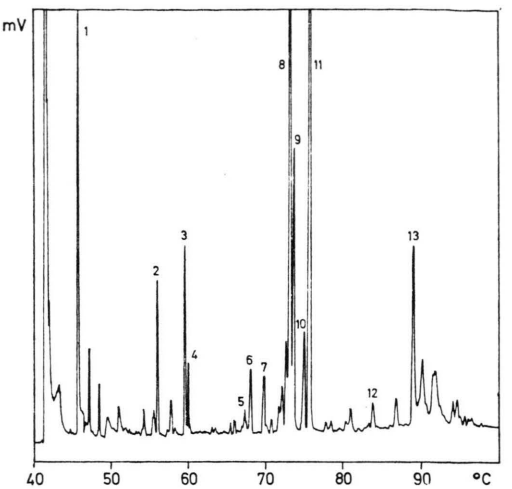
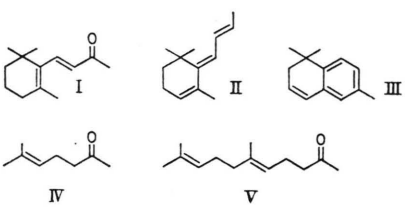


Fig. 1. Gas chromatographic separation of the trapped volatile compounds of Cyanidium on a glass capillary column OV-101 (40–180 °C, 5 °C/min, H_2 carrier gas). 1) 6-methyl-5-hept-2-ene, 8) 1,2-dihydro-1,1,6-trimethylnaphthalene, 11) 6-(but-2-enylidene)-1,5,5-trimethylcyclohex-1-ene, 13) β-ionone.

of the hydrogenated derivatives to be determined. The original and hydrogenated compounds were spiked with reference substances and found to be identical on a capillary column. These compounds were always major constituents of the odorous extracts. The amount of an additional compound which can not be unequivocally correlated to a peak on the chromatogram chart presented changed



markedly during the course of culture. This substance exhibited retention times and a mass spectrum (*m/e* 43(100%), 69(49%), 41(33%), 151(10%), 136(10%), 44(10%), 149(8%), 125(8%), 107(8%), 93(8%), 83(8%), 73(8%), 67(8%), 55(8%), 53(8%), 40(8%), 39(8%), 29(8%)) similar to those of an authentic sample of geranylacetone (**V**). In the literature a spectrum was reported by Buttery and Seifert [20]. Geranylacetone could be superimposed with the algal product on a capillary column. For the identification of the compounds 8 and 11 on the gas chromatogram, these were separated by preparative gas chromato-

Table I. Effect of nor-carotenoids on the growth of algae for 24 hours after the addition of various amounts (ppm) of the stated compounds, given as doubling time (h). ∞ = lethal for algal cells.

Compound		β-Ionone				Methylheptenone				Geranylacetone				Dihydrotrimethyl-naphthalene			
Added amounts [ppm]	0	5	10	50	100	5	10	50	100	5	10	50	100	5	10	50	100
Synechococcus	16	16	15	46⁺	∞	20	20	20		18	20	34⁺	∞	17	52	∞	∞
Anabaena	24	26	22	58	∞	20	21	19	22	23	17	36	∞	24	26	18	39
Nannochloris	16	15	15	14⁺	∞	15	15		16	16	19	24⁺	∞	16	16⁺	16⁺	∞
Cyanidium	23	24	24	∞	∞	22	26	26	32	23	42	∞	∞	24	∞	∞	∞

⁺ Alga treated thus exhibited unchanged growth rate for 24 h, however after this time further growth ceased.

graphy on a packed column (OV-17). They were eluted in reverse sequence. Fraction peak 8 revealed a UV_{max} 274 nm in diethyl ether and the mass spectrum (*m/e* 157(100%), 142(40%), 172(28%), 141(18%), 158(17%), 115(10%), 73(10%), 91(8%), 143(7%), 128(7%), 44(7%)). The m^+ ion exhibited lower intensity as reported by Stoltz *et al.* [21] of 1,2-dihydro-1,1,6-trimethylnaphthalene (**III**). After hydrogenation, the mass spectrum of ionene [22] (1,2,3,4-tetrahydro-1,6,6-trimethylnaphthalene *m/e* 159(100%), 174(19%), 160(15%), 131(8%), 128(8%), 129(7%), 144(6%), 115(6%), 105(6%), 143(5%), 91(5%), 41(5%)) was obtained. Synthesized samples revealed the same spectra and were superimposed. Fraction peak 11 had an UV_{max} 274 in diethyl ether and the mass spectrum (*m/e* 161(100%), 105(99%), 176(44%), 119(42%), 120(34%), 133(28%), 121(24%), 91(24%), 55(19%), 77(16%), 41(16%)). A synthesized sample of (6Z,2E)-6-(but-2-enylidene)trimethylcyclohex-1-ene (**II**) exhibited similar mass and UV spectra. No separation was noticed when the synthesized compound was spiked with the fraction containing peak 11. Besides these major components several different compounds could be noticed. But the amounts available were so small that they were lost either after preparative gas chromatographic separation or microchemical derivatisation. Their mass spectra however indicate that these could be as yet unknown nor-carotenoids.

The physiological activities of the compounds found were determined in an algal bioassay, using *Cyanidium caldarium*, *Anabaena variabilis*, *Synechococcus* 6911 and *Nannochloris coccoides*. The last two species proved especially favourable as test organisms for photometric determinations since the cells exhibit small dimensions, do not clump or stick to the glass walls of the culture vessels, do not foam and can therefore be easily cultivated. To thin cultures exhibiting only slightly declined logarithmic growth different amounts of the test substances were added and the growth determined after 8, 24, 48 and 62 hours. Table I presents the growth rates stated as doubling time in hours. Methylheptenone exhibited only weak effects on the growth rate. The lower threshold for β-ionone, geranylacetone and dihydrotrimethylnaphthalene was near to 10 ppm. The response of *Cyanidium* to the added compounds was expressed by an immediate decline in the growth rate which was essentially dependent on the concentration of compound added. However in *Nannochloris* and *Synechococcus* growth was

Table II. Effect of nor-carotenoids on the growth of algae. (−) no effect on growth, (+) growth is inhibited, (∞) lethal for algal cells.

Compound	β-Ionone				Methylheptenone				Geranylacetone				Dihydrotrimethyl-naphthalene			
Added amounts [ppm]	5	10	50	100	5	10	50	100	5	10	50	100	5	10	50	100
Synechococcus	−	−	+	∞	−	−	−	+	−	−	+	∞	−	+	∞	∞
Anabaena	−	−	+	∞	−	−	−	−	−	−	+	∞	−	−	−	+
Nannochloris	−	−	+	∞	−	−	−	−	−	−	+	∞	−	+	+	∞
Cyanidium	−	−	∞	∞	−	−	−	+	−	+	∞	∞	−	∞	∞	∞

inhibited only 24 h after addition of these compounds. A compilation of the effects of the tested substances on growth can be seen in Table II.

Discussion

Preliminary experiments have demonstrated a great number of volatile odorous compounds liberated from *Cyanidium* into the medium. Some of these have also been found in Cyanobacteria and Chlorophyceae. The analysis in the latter classes is complicated by the heavy contamination with branched and unbranched hydrocarbons. *Cyanidium* however liberates only small amounts of these alkanes and thus this alga presented an ideal opportunity for a detailed study on this subject. The odorous compounds can be obtained in a virtually pure form by the stripping method used. The analysis and identification of the major products showed that all those so far determined belong to nor-carotenoid type molecules. Monoterpenes, characteristic for higher plants have not been found in this study. In this respect the Rhodophycean alga *Cyanidium* [23] resembles the Cyanobacteria, in which up to now no genuine monoterpenes have been found [12]. The fact that α-ionone has not been detected is in agreement with the experiments of Sanderson *et al.* [24] demonstrating that nor-carotenoids originate from carotenes, and of Allen *et al.* [25] who detected only β-carotene in *Cyanidium*. The occurrence of butenylidenetrimethylcyclohexene does not provide opposing evidence for this postulate since the movement of double bonds from the β- to the α-position in the molecule is frequently encountered during the dehydration of β-ionol under a variety of experimental conditions. Dihydrotrimethylnaphthalene is formed *in vitro* when β-ionone epoxide is treated in acid [26]. In fact, the medium of *Cyanidium* has an acid reaction (pH 2 – 3) but no indication was found for the existence of such a precursor molecule during our investigation. Methylheptenone and geranylacetone are the most likely degradation products of open chain carotenes. Though open chain carotenes have not yet been reported in *Cyanidium*, their existence in this organism can be deduced from the general biosynthetic pathway of carotenes in other organisms, the assumption of which should also be valid for *Cyanidium*. The formation of nor-carotenoids in *Cyanidium* resembles the pathways operating in flowers and fruits of higher plants. Butenylidenetrimethylcyclohexene has been found in the passion fruit [27], dihydrotrimethylnaphthalene in peach fruit [21], β-ionone, methylheptenone and geranylacetone in a great variety of fruits. Methylheptenone has also been found in fungi [28] but, nevertheless, there are no reports of these compounds in bacteria. There is some suggestive evidence that nor-carotenoids are generally distributed throughout the major algal classes, *e. g.* β-cyclocitral has been found in the Cyanobacterium *Microcystis* [12] and β-ionone in the Chlorophyceae *Trentepohlia* [3].

This study demonstrated that nor-carotenoids have an effect on photoautotrophic organisms. The threshold values of their efficiency in effecting growth was similar for all algae tested despite their phylogenetic separation. With the exception of methylheptenone the compounds assayed revealed an inhibition between 10 and 50 ppm. It is well known that nor-carotenoids are extremely effective as growth modifiers. Firn *et al.* [29] described the inhibitory properties of a compound on the growth of higher plants which was found to be xanthoxin. Methylheptenone has a stimulatory effect on the germination of uredospores of several species of rust as has β-ionone [30]. Zajic and Kuehn [31] demonstrated a greater antimicrobial activity of β-ionone against soil bacteria than fungi. However, concentrations as high as 2000 ppm were used. In several studies β-ionone was demonstrated to effect carotene biosynthesis without being metabolized itself. Methylheptenone has been shown to inhibit, β-ionone to activate carotene biosynthesis [32, 33]. In natural waters Sakevich [34] has demonstrated the liberation of volatile inhibitory compounds from natural blooms of Cyanobacteria. Their formation was dependent on the physiological state of the algae and on the growth environment. Though their chemical nature was not determined they may have well been nor-carotenoids. An effect of nor-carotenoids under natural conditions is not ruled out, but the concentrations reached may not be high enough to cause a short term inhibition of organisms in natural waters. In these natural conditions a long term inhibition is a much more likely event.

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[1] H. Liebmann, Münch. Beitr. Abwasser-, Fisch- u. Flußbiol. **12,** 127 (1965).
[2] E. G. Bellinger, Proc. Soc. Wat. Treat. Exam. **18,** 106 (1969).
[3] J. Tischer, Hoppe-Seyler's Z. physiol. Chem. **243,** 103 (1936).
[4] N. N. Gerber and H. A. Lechevalier, Appl. Microbiol. **13,** 935 (1965).
[5] R. S. Safferman, A. A. Rosen, C. I. Mashni, and M. E. Morris, Environ. Sci. Techn. **1,** 429 (1967).
[6] N. N. Gerber, Tetrahedron Lett. **1968,** 2971.
[7] J.-A. L. Tabachek and M. Yurkowski, J. Fish. Res. Board Can. **33,** 25 (1976).
[8] R. P. Collins and K. Kalnins, Lloydia **28,** 52 (1965).
[9] R. P. Collins and K. Kalnins, J. Protozool. **13,** 435 (1966).
[10] R. Liersch, Arch. Microbiol. **107,** 353 (1976).
[11] V. Herrmann and F. Jüttner, Anal. Biochem. **78,** 365 (1977).
[12] F. Jüttner, Z. Naturforsch. **31 c,** 491 (1976).
[13] A. Kuhl and H. Lorenzen, Methods in Cell Physiology (D. M. Prescott, ed.) **Vol. 1,** p. 159, Academic Press, London 1964.
[14] M. B. Allen, Arch. Mikrobiol. **32,** 270 (1959).
[15] F. Jüttner, H. Victor, and H. Metzner, Arch. Microbiol. **77,** 275 (1971).
[16] F. Jüttner, The Biology of Blue-Green Algae (N. G. Carr and B. A. Whitton, eds.), p. 536, Blackwell Sci. Publ. Oxford 1973.
[17] P. Karrer and P. Ochsner, Helv. Chim. Acta **31,** 2093 (1948).
[18] J. Barjot, G. Bony, G. Dauphin, P. Duprat, A. Kergomard, and H. Veschambre, Bull. Soc. Chim. France **1973,** 3187.
[19] A. F. Thomas, B. Willhalm, and R. Müller, Org. Mass Spectrom. **2,** 223 (1969).
[20] R. G. Buttery and R. M. Seifert, J. Agr. Food Chem. **16,** 1053 (1968).
[21] L. P. Stoltz, T. R. Kemp, W. O. Smith, Jr., W. T. Smith, Jr., and C. E. Chaplin, Phytochemistry **9,** 1157 (1970).
[22] T. R. Kemp, L. P. Stoltz, and L. V. Packett, Phytochemistry **10,** 478 (1971).
[23] T. Hase, S. Wakabayashi, K. Wada, H. Matsubara, F. Jüttner, K. K. Rao, I. Fry, and D. O. Hall, FEBS Letters **96,** 41 (1978).
[24] G. W. Sanderson, H. Co, and J. G. Gonzales, J. Food Sci. **36,** 231 (1971).
[25] M. B. Allen, T. W. Goodwin, and S. Phagpolngarm, J. Gen. Microbiol. **23,** 93 (1960).
[26] K. L. Stevens, R. Ludin, and D. L. Davis, Tetrahedron **31,** 2749 (1975).
[27] F. B. Whitfield, G. Sugowdz, and D. J. Casimir, Chem. Ind. **1977,** 502.
[28] E. Sprecher, K.-H. Kubeczka, and M. Ratschko, Arch. Pharm. **308,** 843 (1975).
[29] R. D. Firn, R. S. Burden, and H. F. Tayler, Planta **102,** 115 (1972).
[30] R. C. French, A. W. Gale, C. L. Graham, F. M. Latterell, C. G. Schmitt, M. A. Marchetti, and H. W. Rines, J. Agric. Food Chem. **23,** 766 (1975).
[31] J. E. Zajic and H. H. Kuehn, Can. J. Microbiol. **7,** 119 (1961).
[32] T. W. Goodwin, Encyclopedia of Plant Physiology (W. Ruhland, ed.), **Vol. 10,** p. 186, Springer Verl. Heidelberg.
[33] A. P. Eslava, M. I. Alvarez, and E. Cerdá-Olmedo, Eur. J. Biochem. **48,** 617 (1974).
[34] A. I. Sakevich, Hydrobiol. J. (russ.) **9,** 12 (1973).