Phytochemistry, Vol. 24, No. 4, pp. 733–737, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

BIOSYNTHESIS OF UNUSUAL ACYCLIC ISOPRENOIDS IN THE ALGA *BOTRYOCOCCUS BRAUNII*

FRED R. WOLF*, ESTHER K. NEMETHY, JONATHAN H. BLANDING† and JAMES A. BASSHAM

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720, U.S.A.

(*Revised received* 31 *July* 1984)

Key Word Index—*Botryococcus braunii*; Chlorophyceae; alga; biosynthesis; acyclic isoprenoids; botryococcenes.

Abstract—A 'resting state' isolate of the hydrocarbon-producing alga *Botryococcus braunii* photoassimilated sodium [^{14}C]bicarbonate at rates comparable to fast growing algae, such as *Chlorella* (> 150 μg atoms ^{14}C/mg chlorophyll · hr). Early in the reaction (up to several min), most of the radioactivity was associated with water-soluble metabolites. However, labelling of hexane-soluble compounds steadily increased from *ca* 3% at 15 sec to over 50% of the total incorporated ^{14}C at 60 min. The purified hexane fraction, which consisted of a series of botryococcenes and squalene, constituted a relatively constant proportion (40–45%) of the total hexane-soluble radioactivity at all but the earliest time points (< 60 sec). This fraction initially consisted almost exclusively of a C_{30} botryococcene (*ca* 91%) and squalene (*ca* 8%); however, small amounts of radioactivity sequentially appeared in the C_{31}, C_{32} and C_{34} botryococcenes. The results of pulse–chase experiments implicated the C_{30} botryococcene as the precursor of the higher homologues; during the chase, loss of radioactivity from the C_{30} compound was accompanied by a concomitant increase in the labelling of the C_{31} and C_{32} compounds. This study provides further evidence that the relatively slow growth of *Botryococcus* in culture may result, in part, from the diversion of a large proportion of reduced carbon into energetically expensive compounds and that the slower growth rate in the 'resting state' cannot be totally attributed to an impaired or intrinsically slow metabolism.

INTRODUCTION

The colonial green alga *Botryococcus braunii* exists in at least two possibly interconvertible physiological states that produce and accumulate large amounts of different classes of hydrocarbons. 'Active state' colonies synthesize linear olefins, primarily C_{27}, C_{29} and C_{31} dienes [1, 2], which may constitute up to 36% of the dry wt [3]. In contrast, 'resting state' colonies produce various mixtures of unusual acyclic isoprenoids (C_nH_{2n-10}, $n = 30$–37), which have been reported to constitute up to 86% of the dry wt [4]. To date, five structures of this homologous botryococcene series have been elucidated [5–7] (Fig. 1) and *ca* 12 others have been detected and partially characterized by mass spectroscopy [8].

Interest in *Botryococcus* has historically focused upon its role in the formation of a variety of oil-rich deposits dating from the Ordovician period to the present (see ref. [9] for review and references); particularly noteworthy is the reported occurrence of high levels (0.9 and 1.4%) of a saturated derivative of botryococcene (Fig. 1) in two Sumatran crude oils [10]. The alga has also been proposed as a renewable source of liquid hydrocarbons, largely because it forms massive floating blooms that suggest the potential for large-scale cultivation and efficient harvesting [11–13].

However, these considerations cannot be explored properly due to the serious deficiencies in our basic knowledge of the organism [14]. For example, most laboratory studies have utilized 'active state' cultures that,

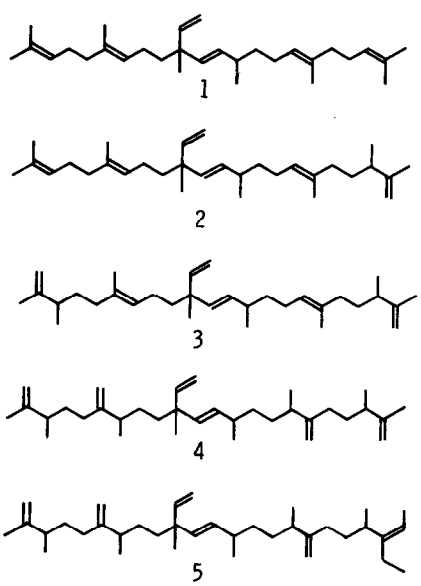

Fig. 1. Structure of botryococcenes elucidated to date: **1**, $C_{30}H_{50}$ [7]; **2**, $C_{31}H_{52}$ [7] (revised structure; E. Casadevall, personal communication); **3**, $C_{32}H_{54}$; **4**, $C_{34}H_{58}$ (botryococcene) [5]; **5**, $C_{36}H_{62}$ [6]. Possible structures have also been proposed for isobotryococcene [6], the principle C_{34} isomer of the Berkeley isolate.

Present addresses: *Martin Marietta Laboratories, 1450 South Rolling Road, Baltimore, MD 21227, U.S.A.; †Zoecon Corp., 975 California Ave., Palo Alto, CA 94303, U.S.A.

in many cases, were isolated decades ago; relatively little is known about the basic biology of the ecologically prevalent 'resting state' form of the alga. The investigation reported here was conducted to provide information on the relationship between general metabolism and the biosynthesis of botryococcene hydrocarbons in a recently acquired 'resting state' isolate of *Botryococcus*.

RESULTS

Hydrocarbons of the Berkeley isolate

The purified hydrocarbon fraction consisted of a mixture of botryococcenes, which varied qualitatively somewhat, and a small amount of squalene (< 2.0%) which, collectively, constituted 25–30% of the colony dry wt. Figure 2 shows a representative chromatogram of a purified extract; squalene was added since it was present in the original extract. Botryococcenes, indicated by asterisks, were identified by GC/MS and by direct comparison to well-characterized samples utilized in previous studies [15]. The remaining compounds were characterized by GC/MS alone. Mass spectral data of the native and hydrogenated compounds indicated that the other members of this series differed from those in Fig. 1 only in the positions of the exomethylene functionality and/or of the internal double bond. For convenience, the C_{32} and C_{33} isomers have been assigned letters based on RR_t. The effects of variable environmental factors on growth and hydrocarbon production will be reported in detail in another publication.

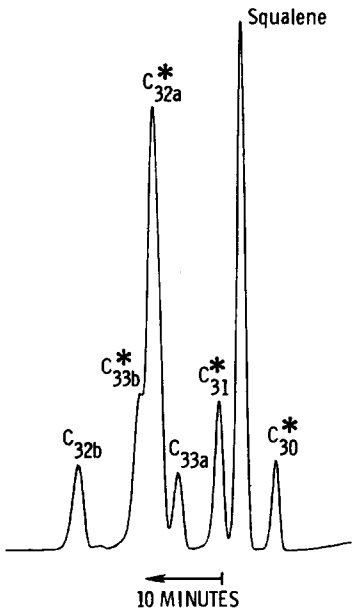

Fig. 2. HPLC separation of the branched isoprenoids contained within the purified hexane extract of the Berkeley isolate of *Botryococcus*. The mobile phase was methanol–methyl cyanide (65:35) at a flow rate of 1.3 ml/min. A mobile phase of methanol–methyl cyanide (80:20) effected the separation of isobotryococcene ($C_{34}H_{58}$) from the C_{32b}–C_{33b} peak complex, but did not satisfactorily separate squalene from C_{31}. Squalene was added to the extract because it was present as a minor constituent in the original extract.

Time-course incorporation of $^{14}CO_2$ into major fractions and individual botryococcenes

Total rates of $^{14}CO_2$ incorporation during time-course experiments usually exceeded 150 μg atoms ^{14}C/mg chlorophyll · hr, with the maximum rate occurring within 15 min. The proportion of radioactivity associated with water-soluble compounds was initially high, but steadily declined after 1 min largely due to increased labelling of hexane-soluble components (Table 1). The incorporation of ^{14}C into insoluble materials (starch, etc.) often varied at early time points (up to 30 sec) between duplicate experiments before exhibiting a stable and similar trend.

At least 98% of the radiolabel of the column-purified hexane fractions was associated with specific botryococcene hydrocarbons and squalene throughout the experiment. These components constituted a large and relatively constant proportion (41.4–43.1%) of the crude hexane fractions after 30 sec (Table 1). The remaining [^{14}C]hexane-soluble constituents were not identified. As can be seen in Table 2, virtually all of the purified hexane-soluble radioactivity was initially associated with the C_{30} botryococcene (85.8%) and with its structural isomer, squalene (10.1%). Label appeared in the C_{31} compound at 30 sec and, subsequently, in the C_{32a} compound (2–5 min) and in isobotryococcene (5 min). Very little activity was associated with the C_{32b} or C_{33} botryococcenes, even at later time points. A general trend is evident in the data shown in Table 2: the percentage of activity associated

Table 1. Time-course incorporation of $^{14}CO_2$ into various fractions extracted from *Botryococcus braunii*

Time	Total incorporation (μg atoms ^{14}C)	Water-soluble (%)	Insoluble (%)	Hexane-soluble (%)	Acyclic isoprenoids (%)	Acyclic isoprenoids as percentage of hexane-soluble fraction
5 sec	1.6	79.3	17.9	2.9	—	—
15 sec	5.1	85.9	11.1	3.0	1.0	32.5
30 sec	12.1	89.7	4.8	4.8	2.1	43.1
60 sec	30.5	74.8	13.6	11.6	4.9	41.8
2.5 min	75.5	57.0	17.4	25.6	10.8	42.1
5 min	159	43.6	21.2	35.2	14.6	41.4
15 min	512	26.2	28.4	45.4	19.1	42.1
30 min	942	17.2	30.4	52.4	22.1	42.1
60 min	1677	13.3	28.7	57.9	28.2	43.0

Table 2. Time-course incorporation of ^{14}C into individual branched isoprenoids (expressed as a percentage of the total purified hexane fraction)

Time	$C_{30}H_{50}$	$C_{31}H_{52}$	$C_{32}H_{54a}$	$C_{34}H_{58}$	Squalene
15 sec	85.8	—	—	—	10.1
30 sec	86.2	1.7	—	—	11.1
60 sec	86.2	2.5	Trace	—	10.2
2.5 min	86.1	1.6	0.3	Trace	11.4
5 min	85.0	1.9	0.4	0.07	11.0
15 min	84.0	2.8	0.8	0.2	11.2
30 min	83.7	3.9	0.8	0.3	10.0
60 min	82.7	5.2	0.8	0.4	9.0

with the C_{30} botryococcene decreases with time while the reverse holds true for the higher homologues; the percentage radioactivity associated with squalene, however, remains relatively constant.

Pulse–chase experiments with $^{14}CO_2$

Pulse–chase experiments were conducted to determine if the C_{30} botryococcene was the precursor to any or all of the higher homologues. Figure 3 depicts the results from an experiment employing a 2.5 min pulse followed by a 5 day chase. Throughout the experiment, the purified hexane extracts contained from 53.2–58.5% of the hexane-soluble ^{14}C, over 98% of it associated with C_{30}, C_{31}, C_{32a} botryococcenes and squalene. Immediately following the pulse (time 0), over 99% of the label distributed among these compounds was associated with the C_{30} botryococcene (90.9%) and squalene (8.5%). Subsequently, activity of the C_{30} botryococcene continuously declined, while labelled C_{31} and C_{32a} increased correspondingly. Labelled C_{31} appeared rapidly at first, reaching a maximum at 3 days and declining slightly thereafter, whereas the initially lower rate of label incorporation into C_{32a} continued approximately unchanged throughout the chase. Very little activity could be confidently ascribed to any of the other botryococcenes.

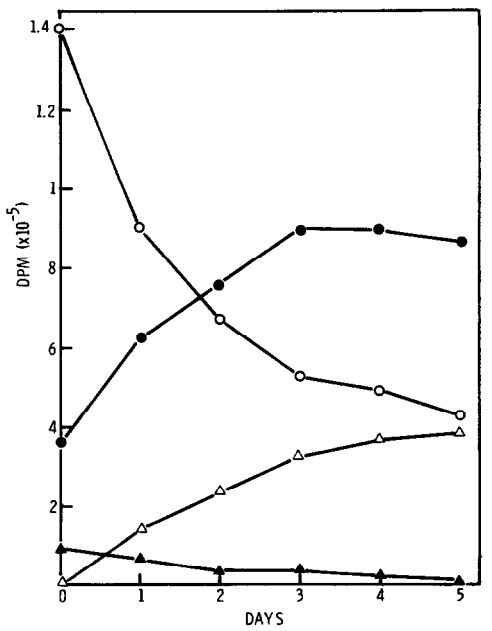

Fig. 3. Radioactivity associated with individual branched isoprenoids during a 5 day pulse–chase experiment: ○, C_{30} botryococcene; ●, C_{31}; △ C_{32b}; ▲, squalene.

Between time 0 and day 1 substantial radioactive flux apparently occurred between the water-soluble and insoluble fractions (data not shown), while little change took place in the total radioactivity of the hexane-soluble and branched isoprenoid fractions. Throughout the remainder of the experiment, however, the radioactivity of all of the fractions remained virtually constant, indicating that the changes observed in Fig. 3 probably occurred within the branched isoprenoid pool and did not result from the input from other (e.g. water-soluble) pools of metabolites.

DISCUSSION

The terms 'active state' and 'resting state' were originally intended to indicate the relative growth potentials of the two principal physiological variants of *Botryoccocus* [4]. However, the growth rate of the Berkeley 'resting state' isolate is comparable to those reported for active state cultures grown under fundamentally similar conditions [3]. If the Berkeley isolate is typical of resting state populations in general (which from existing evidence seems likely), then the only definitive and readily ascertainable distinction between the two forms of the alga at present is the specific type of hydrocarbons they synthesize and store. For this reason, it is suggested that the terms 'active state' and 'resting state' be abandoned in favour of the respective designations L-form (for linear hydrocarbon) and B-form (for branched or botryococcene hydrocarbon).

Culture studies employing conventional growth factors have repeatedly demonstrated the relatively slow growth potential of *Botryococcus*; under optimum growth conditions, the minimum mass doubling time of the Berkeley isolate was *ca* 40 hr (Wolf, F. R., unpublished results) compared to 9 hr for certain species of *Chlorella* [16]. Sluggish growth apparently does not result from impaired diffusion of CO_2 and nutrients to the cells or an intrinsically slow metabolism: the maximum rates of CO_2 incorporation, photosynthetic oxygen evolution [3] and dark respiration [17] are comparable to those reported for *Chlorella*. This anomaly appears to result, in part, from the mechanism that diverts a relatively large proportion of the metabolic energy of the alga into the synthesis of highly reduced and, consequently, energetically expensive compounds [17]. This phenomenon is manifested not only in the composition of the product, but also in the rate at which $^{14}CO_2$ is incorporated into the botryococcenes and other hexane-soluble compounds. Thus, the extraordinary metabolism of *Botryococcus*, as determined by calorimetry, should be taken into account in specifying the growth parameters.

Results obtained from pulse–chase experiments indicate that the C_{30} botryococcene is the precursor of the higher homologues, which probably arise via successive methylations on the C_{30} backbone. The decline in labelled C_{30} botryococcene during the chase corresponded quantitatively to the increase in labelled C_{31} and C_{32a}; indeed, the eventual slight decline in C_{31} activity suggested its conversion to C_{32a}. The lack of radioactivity

associated with the C_{33} and C_{34} compounds was probably related to culture conditions, since aeration of batch cultures with 0.3% CO_2-enriched air, such as those used in these experiments, initially favours the synthesis of the lower botryococcene homologues (C_{30}, C_{31}, C_{32a}). In contrast, cultures aerated with ambient air demonstrate a greatly increased mass doubling time (*ca* 6 days compared to 40 hr) and accumulate higher (C_{33} and C_{34}) homologues; in some instances, isobotryococcene alone constituted over 70% of the total hydrocarbon fraction (Wolf, F. R., unpublished). The results of these experiments suggest that the successive methylations (presumably via *S*-adenosylmethionine) occur at a much slower rate than the synthesis of the basic backbone structure of the lowest homologue, the C_{30} botryococcene.

The only apparent inconsistency in the labelling kinetics of the botryococcenes is the low rate of label incorporation into the C_{32b} compound compared to C_{32a}. No explanation can be offered at this time; however, it is noteworthy that C_{32b} accumulated to significant levels in CO_2-enriched batch cultures only after a lag period of *ca* 1 week and was never a major constituent of ambient air-grown cultures. It is, therefore, possible that the cultures used for the incorporation studies had not attained the state which favours the accumulation of C_{32b}. The solution to this question will probably require structural elucidation of the botryococcenes and more definitive biosynthetic studies.

Data presented in this paper indicate that botryococcene biosynthesis is a major path for carbon utilization in the B-state of this alga and that the botryococcenes are synthesized at a significantly faster rate than squalene. The results of the pulse–chase experiments indicate that the higher homologues are formed from the C_{30} compound, but the possibility of the methylation step at the C_{15} level cannot be conclusively eliminated. This latter model, however, would require drastically higher rates of formation for the C_{30} botryococcene than for the higher homologues; or, alternatively, the model would have to invoke the methylation of the C_{15} isoprenoid pyrophosphate precursor as the rate-limiting step in the overall biosynthetic scheme. Further elaboration of the biosynthesis of botryococcenes is presently under investigation.

EXPERIMENTAL

Isolation and culture. Colonies of *Botryococcus* were isolated by Dr. Arthur M. Nonomura from lily-culturing tanks located in a greenhouse on the campus of the University of California, Berkeley. Attempts to produce axenic cultures using rinsing and dilution techniques alone and in conjunction with antibiotics failed. However, the restricted nature of the bacterial contamination was repeatedly verified via light microscopic inspection and platings on a variety of agarized bacterial media.

Maintenance cultures were grown at 22–24° in 2.5 l. Fernback flasks, continuously bubbled with air and illuminated with cool-white fluorescent tubes (125 $\mu E/m^2 \cdot sec$) on a 16:8 light–dark cycle. The growth medium contained the following components (mg/l. H_2O): $Ca(NO_3)_2 \cdot 4H_2O$ (100), NH_4Cl (26.5), $MgSO_4 \cdot 7H_2O$ (25), K_2HPO_4 (10), H_3BO_3 (0.6), MOPS buffer (3.14), Na_2EDTA (7.7), $ZnCl_2$ (0.624), $CuCl_2 \cdot 2H_2O$ (0.268), $NaMoO_4 \cdot 2H_2O$ (0.252), $CoCl_2 \cdot 6H_2O$ (0.420), $FeSO_4 \cdot 7H_2O$ (2.5), and $MnCl_2 \cdot 4H_2O$ (0.360). The pH of the medium was adjusted to 7.2 before autoclaving.

Extraction, preparation and analysis of botryococcenes. Culture aliquots were filtered, rinsed, dried in a vacuum dessicator for 24 hr and then oven-dried at 60° to constant wt. An int. standard was added to the dried sample and the hydrocarbons were extracted via sonication with hexane. The crude hexane extract was purified by CC on silica gel with hexane as eluant.

GC was performed using a FID instrument fitted with a 30 m × 0.25 mm column containing 5% DB-5 as the stationary phase. The carrier gas was He (2 ml/min) and the split ratio was 9:1. The column temp. was either 240° or 245° and the injection port and FID temps were 300°. GC/MS was performed on a quadropole instrument equipped with a data system.

Time-course of ^{14}C incorporation expts. A 100 ml aliquot of maintenance culture was sterile filtered, resuspended in fresh medium and grown at 22–24° with continuous bubbling (0.3% CO_2 in air). After 2 days, 1 ml aliquots were pre-illuminated for 10 min and the cultures were injected with a soln of $NaH^{14}CO_3$ (sp. act. 52.5 $\mu Ci/\mu g$ atom ^{14}C) to a final concn of 6 mM. At appropriate time intervals (see Results), cells were killed by adding hot MeOH. All radioactive labelling expts were conducted twice with duplicate samples for each time point.

Pulse–chase expts. A 20 ml aliquot of maintenance culture was pre-conditioned for 2 days as described and injected with a soln of $NaH^{14}CO_3$ (sp. act. 52.5 $\mu Ci/\mu g$ atom ^{14}C) to a final concn of 6 mM. After a 2.5 min pulse, the culture was quickly filtered, rinsed with fresh medium and transferred to a new bubbling flask. At appropriate time points after the rinse, duplicate 1 ml aliquots were killed by addition of hot MeOH. The culture flask was then returned to the 'pre-conditioning' growth regime for the duration of the expt. All procedures were carried out under sterile conditions.

Extraction and analysis of products. The killed colony extract was centrifuged and the supernatant removed. The residue was extracted twice with each of MeOH, hexane and MeOH–H_2O (1:1) by vigorous shaking with 5 ml of solvent for 10 min at 50°. The supernatants were dried under a stream of N_2. Hexane (5 ml) and H_2O (5 ml) were added and, after mixing and centrifugation, the aq. layers were extracted twice more with hexane. The hexane extracts were reduced to a suitable vol. and an aliquot was removed for ^{14}C determination by scintillation counting. An aliquot of the aq. phase was dried under N_2, acidified with HOAc and suspended in Aquassure for scintillation counting. The insoluble residue was combusted in an oxidizer and then counted.

HPLC separation of branched hydrocarbons. The silica gel column purified hexane extracts were resolved into individual components by HPLC. Baseline resolutions of all major components were obtained using two coupled 25 cm × 4.6 mm i.d. Altex Ultrasphere ODS columns, with either 4:1 or 13:7 MeOH–MeCN as the mobile phase at a flow rate of 1 or 1.3 ml/min. The detection method was with UV light at 215 nm. Squalene and the C_{31} compound could be well separated by the 13:7 mobile phase (Fig. 2), whereas the C_{32b}–C_{33b} complex and isobotryococcene ($C_{34}H_{58}$) were resolved only by the 4:1 mobile phase. In this latter phase, squalene was observed only as a shoulder. Over 98% of the applied radioactivity was recovered during each radioactive analysis. The correspondence between radioactivity and mass peaks was excellent for all cases; only background radioactivity levels were observed at baseline.

Chlorophyll determination. For chlorophyll determination, a culture aliquot was filtered, rinsed with H_2O and homogenized with Me_2CO and alumina. The mixture was then centrifuged, homogenized, recentrifuged and decanted. The pellet was re-extracted with Me_2CO for 2 hr with stirring and the combined extracts were centrifuged in Me_2CO–H_2O (4:1) at 1000 *g* for 10 min. *A* was measured at 645 and 663 nm, and total chlorophyll was estimated using the equations of Bruinsma [18].

Acknowledgements—We wish to thank Dr. Arthur Nonomura for providing the isolate of *Botryococcus braunii* used in this study.

This work was supported in part by a grant from SOHIO and, in part, by the Assistant Secretary for Conservation and Renewable Energy, Office of Renewable Energy, Biomass Energy Technologies, Division of the U.S. Department of Energy, under contract No. DE-AC03-76F00098.

REFERENCES

1. Gelpi, E., Oró, J., Schneider, H. J. and Bennett, E. O. (1968) *Science* **161**, 700.
2. Knights, B. A., Brown, A. C., Conway, E. and Middleditch, B. S. (1970) *Phytochemistry* **9**, 1317.
3. Largeau, C., Casadevall, E., Dif, D. and Berkaloff, C. (1980) *Energy from Biomass* (Palz, W., Chartier, P. and Hall, D. O., eds.) First E.C. Conference. Applied Science Publishers, London.
4. Brown, A. C., Knights, B. A. and Conway, E. (1969) *Phytochemistry* **8**, 543.
5. Cox, R. E., Burlingame, A. L., Wilson, D. M., Eglinton, G. and Maxwell, J. R. (1973) *J. Chem. Soc. Chem. Commun.* 284.
6. Galbraith, M. N., Hillen, L. W. and Wake, L. V. (1983) *Phytochemistry* **22**, 1441.
7. Metzger, P. and Casadevall, E. (1983) *Tetrahedron Letters* **24**, 4013.
8. Wake, L. V. and Hillen, L. W. (1981) *Aust. J. Mar. Freshwater Res.* **32**, 353.
9. Cane, R. F. (1977) *Trans. R. Soc. S. Aust.* **101**, 153.
10. Moldowan, J. W. and Siefert, W. K. (1980) *J. Chem. Soc. Chem. Commun.* 912.
11. Hillen, L. W. and Warren, D. R. (1976) Report ME 284. Aeronautical Research Laboratories, Melbourne, Australia.
12. Hillen, L. W., Pollard, G., Wake, L. V. and White, N. (1980) MRL-R-783. Materials Research Laboratories, Melbourne, Australia.
13. Wake, L. V. and Hillen, L. W. (1980) *Biotech. Bioeng.* **22**, 1637.
14. Wolf, F. R. (1983) *Appl. Biochem. Biotech.* **8**, 249.
15. Wolf, F. R. (1981) Ph.D. Thesis, Texas A & M University.
16. Myers, J. (1953) in *Algal Culture From Laboratory to Pilot Plant* (Burlew, J. S., ed.), p. 37.
17. Belcher, J. H. (1968) *Arch. Mikrobiol.* **61**, 335.
18. Bruinsma, J. (1963) *Photochem. Photobiol.* **2**, 241.