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**Author**

Wolf, F.R.

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# CHEMICAL BIODYNAMICS DIVISION

BIOSYNTHESIS OF UNUSUAL ACYCLIC ISOPRENOIDS IN THE ALGA Botryococcus braunii

F.R. Wolf, E.K. Nemethy, J.H. Blanding,
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## DISCLAIMER

# BIOSYNTHESIS OF UNUSUAL ACYCLIC ISOPRENOIDS
## IN THE ALGA <u>BOTRYOCOCCUS BRAUNII</u>

Fred R. Wolf[1], Esther K. Nemethy, Jonathan H. Blanding[2] and James A. Bassham
Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory
University of California
Berkeley, CA  94720


1.  Present Address:  Martin Marietta Laboratories
                      1450 South Rolling Road
                      Baltimore, MD 21227



2.  Present Address:  Calgene, Inc.
                      1910 Fifth Street
                      Davis, CA 95616

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ABSTRACT

A "resting state" isolate of the hydrocarbon-producing alga Botryococcus braunii photoassimilated $[^{14}C]HCO_3$ 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 timepoints (<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 homologs; during the chase, loss of radioactivity from the $C_{30}$ compound was accompanied by a concomittant increase in the labeling 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 <u>Botryococcus braunii</u> 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 $C_{27}$, $C_{29}$ amd $C_{31}$ dienes (1,2), which may

constitute up to 36% of the dry weight (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

weight (4). To date, five structures of this homologous botryococcene series

have been elucidated (5-7) (Fig. 1) and about 12 others have been detected and

partially characterized by mass spectroscopy (8).

Interest in <u>Botryococcus</u> has historically focused upon its role in the

formation of a variety of oil-rich deposits dating from the Ordivician period

to the present (see 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,

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

This investigation 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.

## EXPERIMENTAL

Isolation and culture of Botryococcus.   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 Fernbach flasks
continuously bubbled with air and illuminated with cool-white fluorescent
tubes (125 $\mu E/m^2/sec^1$) on a 16:8 LD cycle.  The growth medium contained the
following components  (mg/l $H_2O$):  $Ca(NO_3)_2$ $4H_2O$ (100), $NH_4Cl$ (26.5),
$MgSO_4$ $7H_2O$ (25), $K_2HPO_4$ (10), $H_3BO_3$ (0.6), MOPS buffer (3.14), $Na_2EDTA$ (7.7),
$ZnCl_2$ (0.624), $CuCl_2$ $2H_2O$ (0.268), $NaMoO_4$ $2H_2O$ (0.252), $CoCl_2$ $6H_2O$ (0.420),
$FeSO_4$ $7H_2O$ (2.5), and $MnCl_2$ $4H_2O$ (0.360).  The last seven ingredients were
added as a single 1000-fold concentrated stock solution prepared by boiling
the $Na_2EDTA$ in 800 ml $H_2O$ for 5 min, adding the salts, and bringing the final
volume to 1 l.  The pH of the medium was adjusted to 7.2 before autoclaving.

Extraction, preparation, and analysis of botryococcenes.  Culture aliquots
were filtered, rinsed, and placed in an evacuated dessicator for 24 hr before
being oven dried at 60° to a constant weight.  Dried samples were placed in 20

ml glass vials along with an internal standard, and extracted via sonication for 30 min each in three 15 ml changes of hexane; botryococcenes were not present at significant levels in residues subjected to an additional 8 hr Soxhlet extraction with hexane. The crude hexane extracts were combined, evaporated under nitrogen to about 0.1 ml, and purified by column chomatography on silica gel with hexane as the elutant.

GLC was performed using an FID-equipped instrument fitted with a 30 m x 0.25 mm column containing 5% DB-5 as the stationary phase. The carrier gas was He (2ml/min) and the split ratio 9:1. The column temp. was either 240° or 245° and the injection port and FID temperatures were 300°. GC-MS was performed on a Finnegan 4000 instrument, equipped with an Incos Data System.

Time-course [$^{14}$C] incorporation experiments. A 100 ml aliquot of maintenance culture was sterile filtered, resuspended in fresh medium, and grown in a 250 ml Erlenmeyer bubbling flask with continuous bubbling (0.3% $CO_2$ in air) and illumination (250 $\mu$E/m$^2$/sec) at 22-24°. After two days, 1 ml aliquots were dispensed into 5 ml Erlenmeyer flasks and preilluminated while shaken for 10 min. The flasks were then capped with a serum stopper and the culture injected with a solution of [$^{14}$C]NaHCO$_3$ (sp. act. 52.5 $\mu$Ci/$\mu$g atom [$^{14}$C]) to a final concentration of 6 mM. At appropriate time intervals (see RESULTS), cells were killed by the addition of 4 ml of hot MeOH. All radioactive labeling experiments were conducted twice with duplicate samples for each timepoint.

Pulse-chase experiments. A 20 ml aliquot of maintenance culture was dispensed into a 50 ml Erlenmeyer bubbling flask, preconditioned for 2 days as described, and injected with a solution of $[^{14}C]NaHCO_3$ (sp. act. 52.5 $\mu$Ci/ $\mu$g atom $[^{14}C]$) to a final concentration of 6 mM. After a 2.5 min pulse, the culture was quickly filtered and rinsed with 250 ml of fresh medium, and a 20 ml aliquot was transferred to a new 50 ml bubbling flask. At appropriate timepoints after the rinse, duplicate 1 ml aliquots were killed by addition of hot MeOH. The culture flask was then returned to the "preconditioning" growth regime for the duration of the experiment. All procedures were carried out under sterile conditions.

Extraction and analysis of products. The killed colony extract was transferred with several hot MeOH rinses to conical tubes and centrifuged, and the supernatant was removed. Since preliminary experiments demonstrated cells to be recalcitrant to extraction, the residue was extracted twice each with MeOH, hexane, and 1:1 MeOH-$H_2O$. For each extraction, the residue was shaken vigorously with 5 ml with hot solvent for 10 min in a water bath maintained at 50°. All six supernatants were combined and reduced to near dryness under a stream of $N_2$. Five ml aliquots each of hexane and $H_2O$ were added, and the solutions were agitated on a vortex mixer and centrifuged to better facilitate phase separation. The hexane layer was removed and the aq. fraction was extracted twice more with hexane. The hexane extracts were combined and

reduced to a suitable volume, and an aliquot was removed for radiocarbon determination by scintillation counting. An aliquot of the aq. phase was dried under $N_2$, acidified with HOAc and suspended in 0.5 ml of $H_2O$ and 5.5 ml of Aquassure (New England Nuclear Co., Boston, MA) for scintillation counting. The insoluble residue was combusted in an oxidizer, and then counted.

HPLC separation of the branched hydrocarbons. The silica gel column-purified hexane extracts were resolved into individual components by high pressure liquid chromatography using a Beckman-Altex instrument. Baseline resolutions of all components were obtained using two coupled 25cm x 4.6 mm i.d. Altex Ultrasphere ODS columns, either with 80:20 or 65:35 MeOH-$CH_3CN$ as the mobile phase at a flow rate of 1 or 1.3 ml/min. The detection method was UV at 215nm. Squalene and the $C_{31}$ compound could be well separated by using the 65:35 mobile phase (Fig.2 ), whereas the $C_{32b}$-$C_{33b}$ complex and isobotryococcene ($C_{34}H_{58}$) were resolved only by using 80:20 mobile phase. Under these latter conditions squalene could be observed only as a shoulder.

Chlorophyll determination. A 5 ml culture aliquot was filtered and rinsed with 1.5 ml of $H_2O$ into a glass homogenizer containing ca 3 ml of $Me_2CO$ and a small amount of alumina as a grinding agent. This mixture was centrifuged, homogenized, and recentrifuged, and the supernatant was removed and stored in the dark at -20°. The pellet was reextracted with 4 ml of $Me_2CO$, placed in a vial and magnetically stirred for 2 hr in the dark at -20°. The volume of the

combined extracts was adjusted to 10 ml of 4:1 $Me_2CO-H_2O$, and they were centrifuged at 1000 g for 10 min. Absorbance was measured at 645 nm and 663 nm, and total chlorophyll was estimated using the equations of Bruinsma (15).

RESULTS

Hydrocarbons of the Berkeley isolate. The purified hydrocarbon fraction
consisted of a mixture of botryococcenes and a small amount of squalene
(<2.0%), which collectively constituted 25-30% of the colony dry weight.
There was some qualitative variation; however, the hydrocarbon fraction
represented by Fig. 2 was typical of those obtained from experimental
cultures. Botryococcenes indicated by asterisks were identified by GC-MS and
by direct comparison to well-characterized samples utilized in previous
studies (16). The remaining compounds were characterized by GC-MS alone. For
convenient reference, the $C_{32}H_{54}$ isomers have been assigned letters based on
retention time. A detailed account of the effects of variable physiochemical
factors on growth and hydrocarbon production is the subject of another
publication.

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 by 15 min. The proportion of radioactivity associated
with water-soluble compounds was initially high, but steadily declined after
60 sec largely due to increased labelling of hexane-soluble components (Table
1). $^{14}C$ incorporation into insoluble materials (starch, etc.) often varied at
early timepoints (up to 30 sec) between identically executed experiments
before exhibiting a stable trend.

HPLC analyses of column-purified hexane extracts indicated that a minimum of 98% of the label 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 $[^{14}C]$-labelled hexane-soluble constituents were not identified. As can be seen in Table 2, virtually all of the purified hexane-soluble radioactivity was 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 $C_{32c}$ 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 timepoints. A general trend is evident in the data shown in Table 2: the percentage of activity associated with the $C_{30}$ botryococcene decreases with time while the reverse holds true for the higher homologs; the % radio-activity 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 homologs. 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 $C_{30}$, $C_{31}$, $C_{32b}$ 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, accompanied by a corresponding increase in labelled $C_{31}$ and $C_{32b}$. Labelled $C_{31}$ appeared rapidly at first, reaching a maximum at 3 days and declining slightly thereafter, whereas the initially lower rate of incorporation into $C_{32b}$ continued throughout the chase. Very little activity could be confidently ascribed to any of the other botryococcenes.

Between time 0 and day 1 substantial radioactive flux apparently occurred between the water-soluble and insoluble fractions, 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 easily
ascertainable distinction between the two forms of the alga at present is the
specific type of hydrocarbons that are synthesized and stored. For this
reason, it is suggested that the terms "active state" and "resting state" be
abandoned in favor of the respective designations L-form (for linear
hydrocarbon) and B-form (for branched or botryococcene hydrocarbon.

Culture studies employing conventional growth parameters 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 (17). 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 (18) are comparable to those reported for
Chlorella. This discrepancy results in part from the alga's ability to divert

a relatively large proportion of metabolism into the synthesis of highly reduced and consequently energetically expensive compounds (18). This phenomenon is manifested not only in composition, but also in the rate at which $[^{14}C]O_2$ is incorporated into the botryococcenes and other hexane-soluble compounds. Thus, the extraordinary composition of Botryococcus should be taken into account by including calorimetric determinations along with other growth parameters.

Results obtained from pulse-chase experiments indicate that the $C_{30}$ botryococcene is the precursor of the higher homologs that may arise via successive methylations on the $C_{30}$ backbone. The decline in labeled $C_{30}$ botryococcene during the chase corresponded quantitatively to the increase in labelled $C_{31}$ and $C_{32b}$; indeed, the eventual slight decline in $C_{31}$ activity suggested its conversion to $C_{32b}$. The lack of radioactivity associated with the $C_{32b}$, $C_{33}$, and $C_{34}$ compounds was probably related to culture conditions, since it has been demonstrated that $CO_2$-enrichment initially favors the synthesis of the $C_{30}$, $C_{31}$, and $C_{32b}$ botryococcenes (Wolf, F.R., unpublished results). The results of these experiments, however, also suggest that the successive methylations (presumably via s-adenosyl-methionine) occur at a much slower rate than the synthesis of the basic backbone structure of the lowest homolog, the $C_{30}$ botryococcene.

The family of botryococcenes represents unusual isoprenoid structures which seem to be unique to this organism. Data presented in this paper indicate that botryococcene biosynthesis is a major path for carbon utilization in the B state of this alga. Several possible biogenetic routes can be

envisioned for the formation of the $C_{30}$ botryococcene. One possibility is the coupling of nerolidyl and farnesyl pyrophosphates to yield the peculiar ethylidine group, the common structural feature of all botryococcenes. Alternately, botryococcene biosynthesis may be analogous to that of squalene, except for the last step, the reduction of presqualene pyrophosphate. Nucleophilic attack by hydride ion (from NADPH) either on presqualene pyrophosphate or the cyclobutyl derivative can give the botryococcene skeleton (Fig. 4 ). If the latter mechanism is the route to botryococcenes, then the preferential biosynthesis of botryococcenes over squalene indicates that this alternate, novel mode of ring opening of presqualene pyrophosphate is prevalent in this organism.

The results of the pulse-chase experiments indicate that the higher homologs are formed from the $C_{30}$ compound, but the possibility of the methylation step at the $C_{15}$ level cannot be conclusively eliminated. This model, however, would require drastically higher rates of formation for the $C_{30}$ botryococcene than for the higher homologs; or, alternately 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.

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

| Time | Total incorporation ( g atoms $^{14}C$) | $H_2O$ soluble (%) | Insoluble (%) | Hexane soluble (%) | Acyclic isoprenoids (%) | Acyclic isoprenoids as % 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 % of the total purified hexane fraction).

| Time | $C_{30}H_{50}$ | $C_{31}H_{52}$ | $C_{32}H_{54}$a | $C_{34}H_{58}$ | 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 |

Fig. 1. Structures of botryococcenes elucidated to date: <u>1</u>, $C_{30}H_{50}$ (7);

<u>2</u>, $C_{31}H_{52}$ (7); <u>3</u>, $C_{32}H_{54}$; <u>4</u>, $C_{34}H_{58}$ (botryococcene) (5);

<u>5</u>, $C_{36}H_{62}$ (6). Possible structures have also been proposed for

isobotryococcene (6), the principle $C_{34}$ isomer of the Berkeley

isolate.


Fig. 2. HPLC separation of the branched isoprenoids contained within

the purified hexane extract of the Berkeley isolate of <u>Botryo-</u>

<u>coccus</u>. The mobile phase was 65:35 MeOH:$CH_3$CN at a flow rate

of 1.3 ml/min. A mobile phase of 80:20 MeOH:$CH_3$CN 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.


Fig. 3. Radioactivity associated with individual branched isoprenoids

during a 5 day pulse-chase experiment: O , $C_{30}$botryococcene;

◐ , $C_{31}$; △ $C_{32b}$; ▲ ,squalene.


Fig. 4. Possible mechanisms for the biosynthesis of the $C_{30}$ botryococcene.
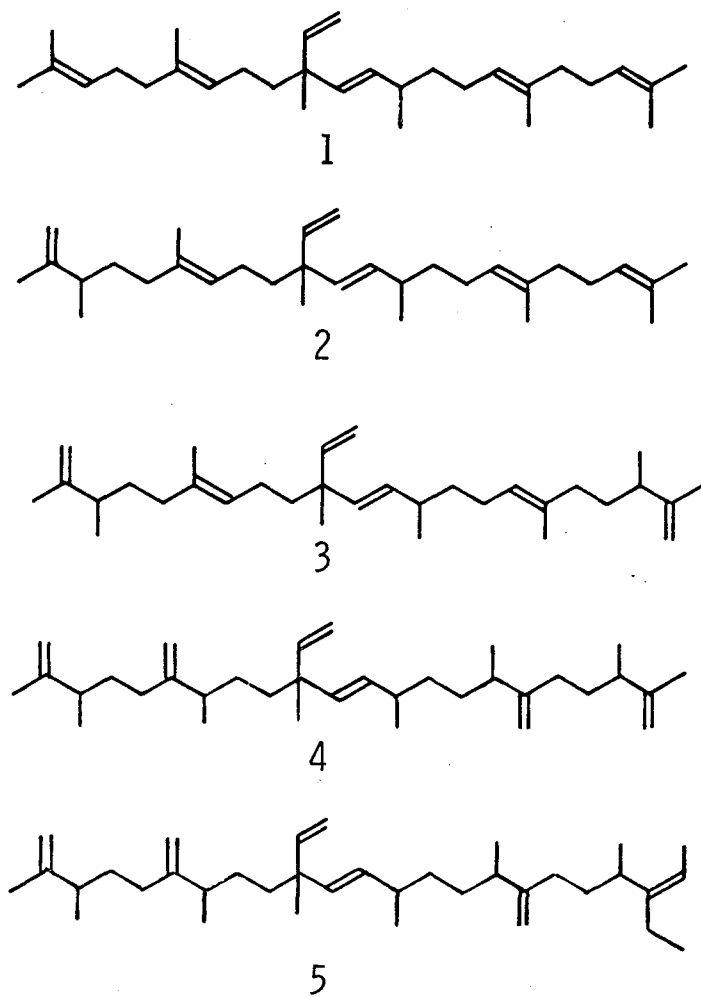
Figure 1.  Wolf, et al., Biosynthesis of Isoprenoids in <u>Botryococcus</u>
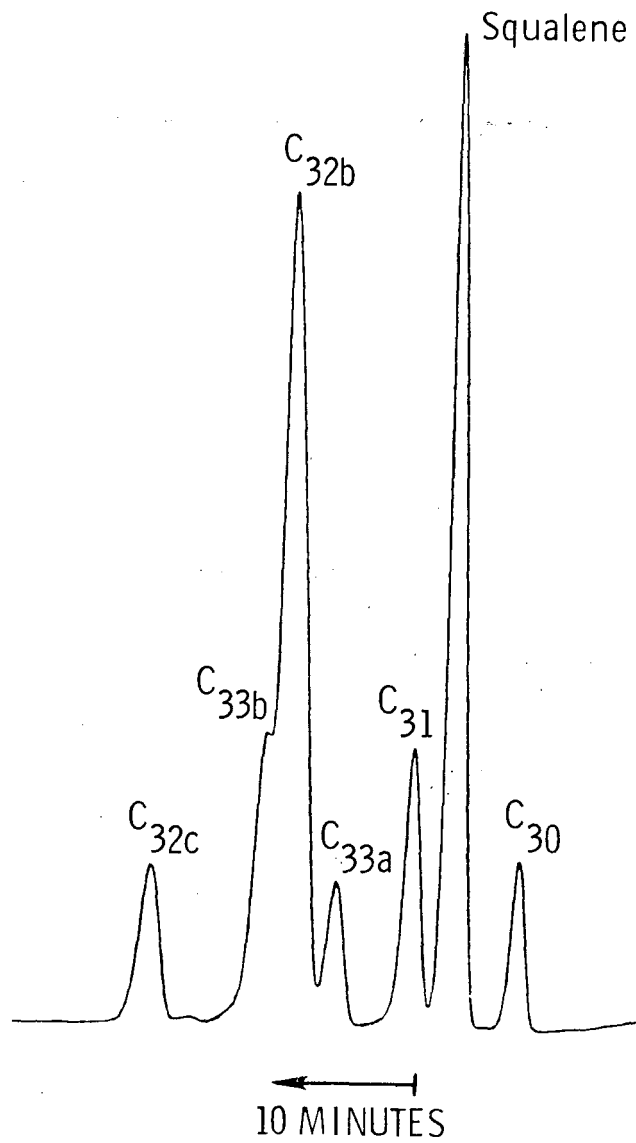
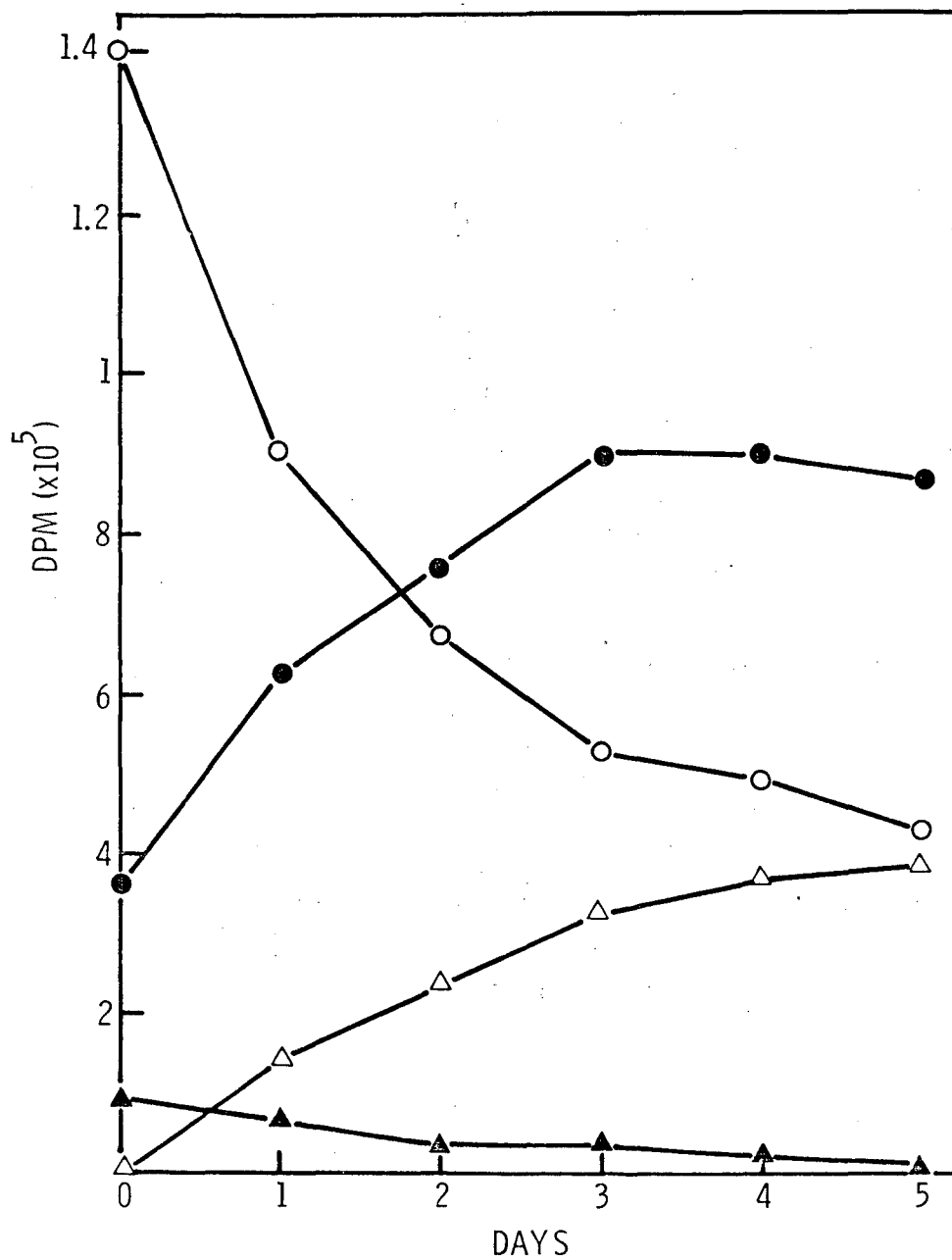Figure 2.  Wolf, et al., Biosynthesis of Isoprenoids in Botryococcus

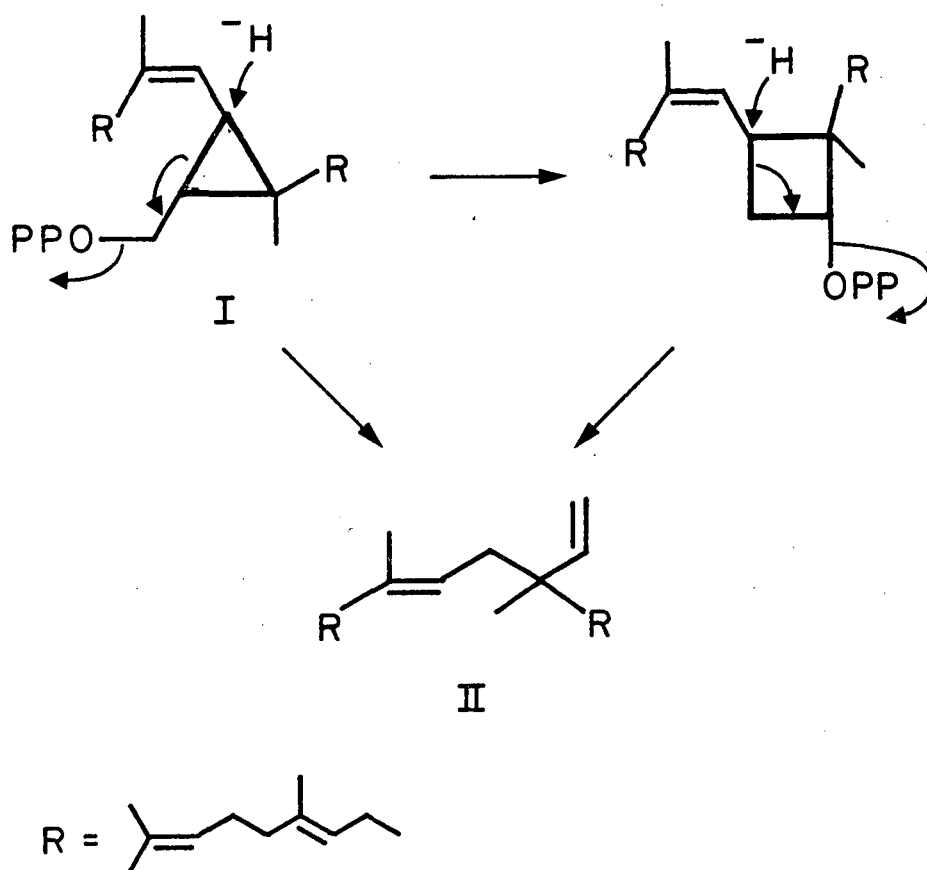Figure 3. Wolf, et al., Biosynthesis of Isoprenoids in Botryococcus

Figure 4. Wolf, et al., Biosynthesis of Isoprenoids in <u>Botryococcus</u>