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ALTERATION OF HYDROXYHOPANONE IN SEDIMENTS

by

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ABSTRACT

In order to identify the diagenetic alteration products of hydroxyhopanone in recently deposited sediments, an oxic and an anoxic sediment were innoculated with a mixture of natural products (isolated from Dammar resin) of which hydroxyhopanone was a major component. The sediments were then incubated for 30 or 60 days at approximately 25°C under oxic and anoxic conditions respectively. Experimental design was such that abiological and biological alteration products and compounds indigenous to the sediment could be distinguished from each other. However, the poison used in the control experiments, $HgCl_2$, failed to work in the anoxic sediment.

At the end of the incubation period the sediments were extracted and the compound classes fractionated by saponification and column chromatography. Four fractions were then analyzed by gas chromatography-mass spectrometry (GC-MS)

The only alteration product of hydroxyhopanone which was found was Hop-22(29)-en-3-one. This dehydration product is believed to have been produced biologically. It was found in the sediments of one of the oxic experiments. Dehydration products of other compounds of the mixture were also found but not identified. No dehydration products

were found in one particularly reducing sediment column, suggesting that hydroxyl groups are preserved under such conditions.

A new compound was found in the chloroform fraction of the innoculated sediments. However, its origin is in doubt because of its presence in an oxic poison control. It may have been present in the starting material and not detected.

The methyl ester of a carboxylic acid was found in the innoculated oxic sediments. This product is reasoned to be produced abiologically. The structure of this compound is unknown.

Two other compounds were found in the anoxic methyl ester fractions. They are, however, apparently not methyl esters. One is believed to be the first component of the starting material. The other is thought to be a 17α(H) epimer of one of the starting materials, possibly hydroxyhopanone. A reaction mechanism is proposed for its formation.

No evidence was found for the formation of 17α(H), 18α(H),21β(H)-bisnorhopane from hydroxyhopanone. This indicates that during early diagenesis hydroxyhopanone does not alter to the bisnorhopane. The precursor to 17α(H),18α(H), 21β(H)-bisnorhopane has not yet been found.

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And last to my wife who provided encouragement and helped me to persevere.

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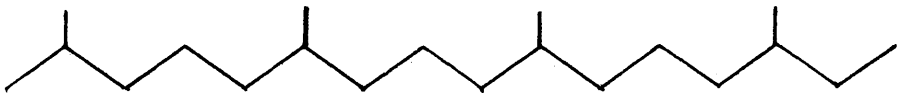
INTRODUCTION

The isolation of porphyrins from shales and crude oils by Treibs in the 1930's (1936) produced the first positive evidence for the biogenic origin of petroleum. Since then much of the literature of organic geochemistry has dealt with the presence of biochemical fossils in petroleum and sediments. Some of these compounds resemble, and presumably are derived from, structures found in living organisms. They have been termed biological markers, and include such types of compounds as isoprenoids, porphyrins, steroids, and terpenoids (Figures 1 and 2). That the precursors to such compounds originated in living tissues seems obvious. Their molecular complexity and high concentrations in geological sources preclude abiogenic origin.

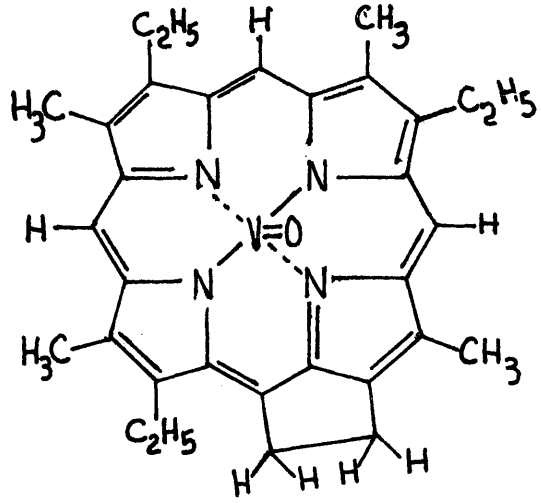
An example of this precursor-product relationship is that of cholesterol and cholestane (Figure 1) Cholesterol is abundant throughout the animal kingdom and has been found to be present in a few lower plants. The biological marker cholestane occurs in ancient sediments and crude oils (Tissot and Welte, 1978, p. 114, and references therein).

Uses of Biological Markers

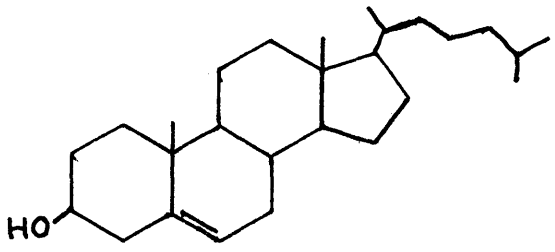
Biological markers are used as guides to the processes by which petroleum and other fossil fuels are formed. Steranes and triterpanes have been applied in this manner.



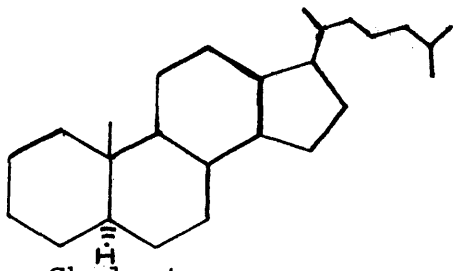
Phytane (Example of an Acyclic Isoprenoid)



Vanadyl Desoxyphylloerythroetioporphyrin
(Example of a Porphyrin)



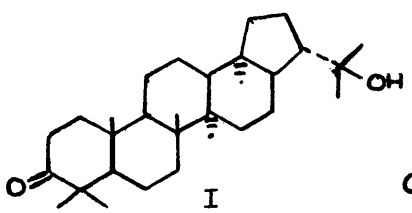
Cholesterol
(Example of a Steroid)



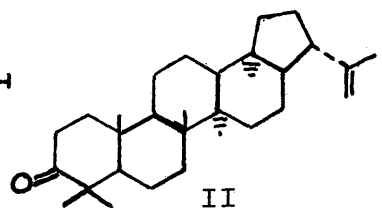
Cholestane
(Example of a Sterane)

Examples of Biological Marker Type Compounds

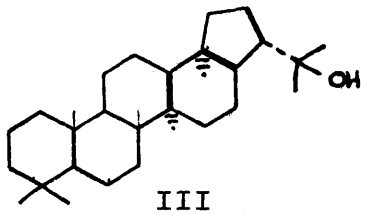
Figure 1



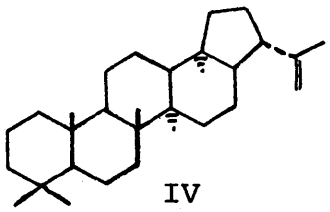
I
Hydroxyhopanone



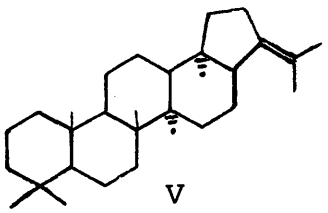
II
Hop-22(29)-en-3-one



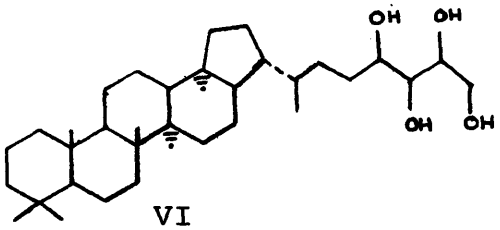
III
22-Hydroxyhopane



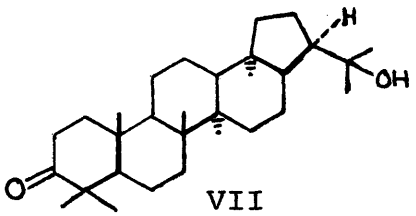
IV
Hop-22(29)-ene



V
Hop-21(22)-ene



VI
Tetrahydroxyhopane



VII
21α(H)-Hydroxyhopanone

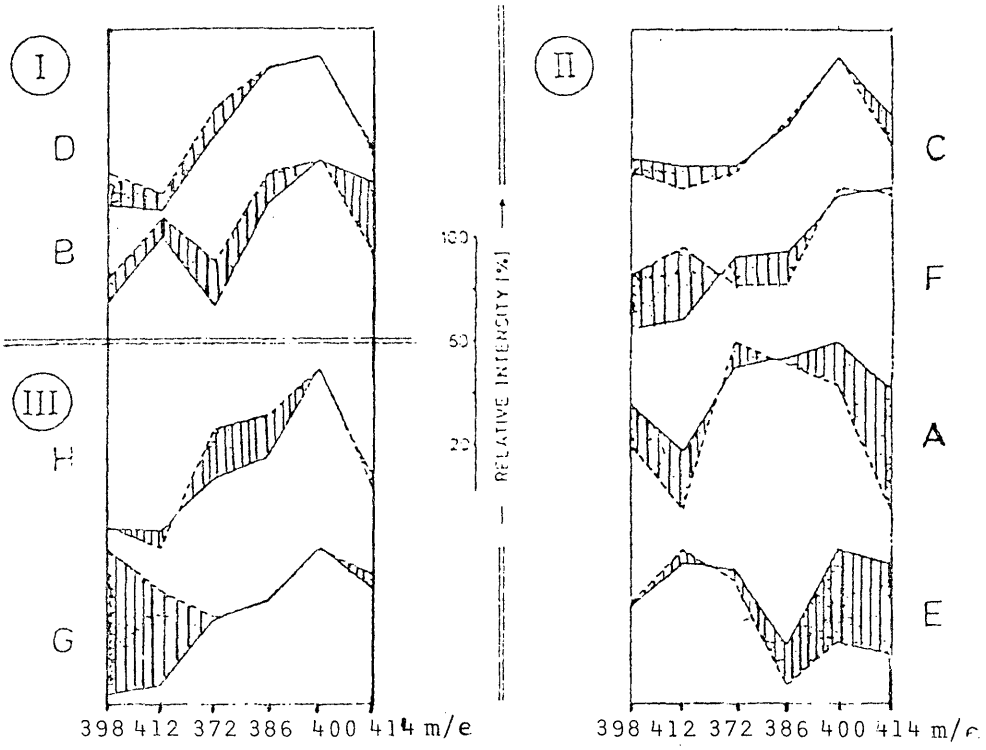
A Few of the Hopanoids Which
Have Been Found in Living Organisms
(Roman numerals are used for natural products)

Figure 2

Seifert (1978a and b and 1977) and Leythaeuser (1977) have used both to correlate oils with the source rocks from which they migrated.

This approach is illustrated by Figure 3, which shows how samples from source rocks and oils may be matched. An explanation of the relationships between the source rocks and oils is given in the caption. One can easily see the pairs which correlate well (ID, IB, and IIC), and those that do not. Pairs which appear to correlate may indicate that the oil may well have migrated from that particular source rock. The correlation is especially good in cases where the source rock and oil exist in the same strata (Figure 3-I), or where they may be close together (Figure 3-II).

Analysis of biological markers from oils and source rocks by gas chromatography-mass spectrometry (GC-MS) characterizes the oils and source rocks on a molecular level. Steranes and triterpanes can be used to fingerprint the hydrocarbons of an oil or source rock because of the specificity of their structure: a difference in stereochemistry about one carbon atom may result in differences in the GC retention time and in the mass spectrum of the molecules being compared. Thus the whole suite of compounds presented is a fingerprint of a particular source rock or oil.



Source Rock-Crude Oil Correlation

Figure 3

(Taken from Leythaeuser, 1977)

Another way in which biological markers may increase our knowledge about the formation of petroleum is that they aid in improving our understanding of the relationship between depositional environments and their associated organic matter; i.e., the types of organic compounds that are deposited and preserved in various settings. Biochemical fossils may enable us to identify the organisms that have contributed to the organic matter of specific depositional environments. Biological markers may better our understanding of the diagenetic pathways which sedimentary organic matter follows by tracing the chemical changes which they undergo. Eventually, we may be able to associate source rocks and fossil fuel reserves with the organisms and the depositional environments that initiated their creation.

Another reason for the importance of biological markers is more academic: they may supply evidence regarding the problem of the origin of life. Biogenic compounds have been found in a one-billion-year-old sediment (Eglington et al., 1964), and in other sediments dating back to the Precambrian (Meinschein et al., 1964) There are organic geochemical data supporting the possibility of life existing in the Archaean (Schopf, 1977). These compounds may also be used as keys to evolutionary developments that living

organisms have undergone since their preservation in the geologic record (Hollerbach and Welte, 1977)

Occurrence of Hopanes in Nature

Pentacylic triterpenoids have been found to be abundant in higher plants and land plants in general. Angiosperms contain a wide variety of triterpenes, while gymnosperms contain relatively few pentacyclic triterpenes. Bryophyta (liverworts and mosses) and Pteridophyta (ferns) contain triterpenes of the hopane skeletal type.

Hydroxyhopanone (I, Figure 2) has been isolated from many Angiosperms. It has been found to be a constituent of the following: pollen grains of the Japanese Silver Birch, Betula platyphylalla (Betulaceae) (Ohmoto et al., 1978); many members of the Dipterocarpaceae family (Bisset et al., 1971); and in Myroxylon balsamium, a member of the Leguminosea family (Wahlberg et al., 1971) 21α(H)-hydroxyhopanone (VII, Figure 2) has been isolated from Lithocarpus cornea of the Fagaceae family (Hui et al., 1976a) Hui (1976b) also found two other 21α(H)-hopanoids in a species of the Myrtaceae family. In nature 21α(H)-hopanoids have generally been found to be subordinate to the regular 21β(H)-hopanoids.

Hop-22(29)-en-3-one (II, Figure 2) has been reported to be a component of the bark of a Himalayan tree (Khastgir

and Pradhan, 1969) Note that this compound is related to I by dehydration.

22-hydroxyhopane (diplopterol, III, Figure 2) and hop-22(29)-ene (diploptene, IV, Figure 2) have been isolated from several families of ferns, such as the Cyatheaeceae (Seshadri and Rangaswami, 1974) Diplopterol has been found in a Japanese fern, Diplopterigium glaucum (Gleicheniaceae) (Ageta et al., 1963a). Hop-22(29)-ene has been isolated from another Japanese fern, Dryopteris crassrhizoma (Aspidaceae) (Ageta et al., 1963b), and from ferns of the Polypodiaceae family (Das Gupta and Khastigir, 1978; Ghisalberti et al., 1970)

Hopenes have been found to be constituents of liverworts and mosses. Hop-21(22)-ene (V, Figure 2) and hop-22(29)-ene have been found in Pseudosceropodium purum (Marsili et al., 1970) Hop-22(29)-ene has also been isolated from Thudium tasmariscifolium (Marsili and Morelli, 1971)

A decade ago it was thought that only higher plants contained triterpenes. Now, however, pentacyclic triterpenes of the hopane skeletal type are known to be abundantly distributed among the more primitive divisions of the plant kingdom, such as Thallophyta (plants characterized by unicellular reproductive systems, such as bacteria, blue-green algae, and lichens). Relatively high concentrations

of hopanes (on the order of 0.5% dry weight) have been found in blue-green algae and bacteria (van Dorsselaer, 1974) such as Acetobacter xylinum (Forster et al., 1973; Rhomer and Ourisson, 1976), Bacillus acidocaldarius (DeRosa et al, 1971, 1973), Methylococcus capsulatus (Bird et al, 1971), Lyngbya aestuarii, Nostoc sp., and Croococcus turgidus (Gelpi et al., 1970). These organisms all have one compound in common: hop-22(29)-ene. Acetobacter xylinum has also been found to contain 22-hydroxyhopane (Bird et al., 1971) and tetrahydroxyhopane (VI, Figure 2), a C-35 hopanol (Forster et al., 1973).

The bacteria above represent a wide variety of environments. A. xylinum grows at temperatures ranging from 5°C to 42°C (30°C optimum). Its pH range is from 4 to 8. At the other end of the temperature scale is B.acidocaldarius, with a range of 45° to 70°C. Its pH range is from 2 to 6. M. capsulatas' temperature range is intermediate between the other two: 30° to 50°C. No pH range was given (Bergey's Manual, 1973).

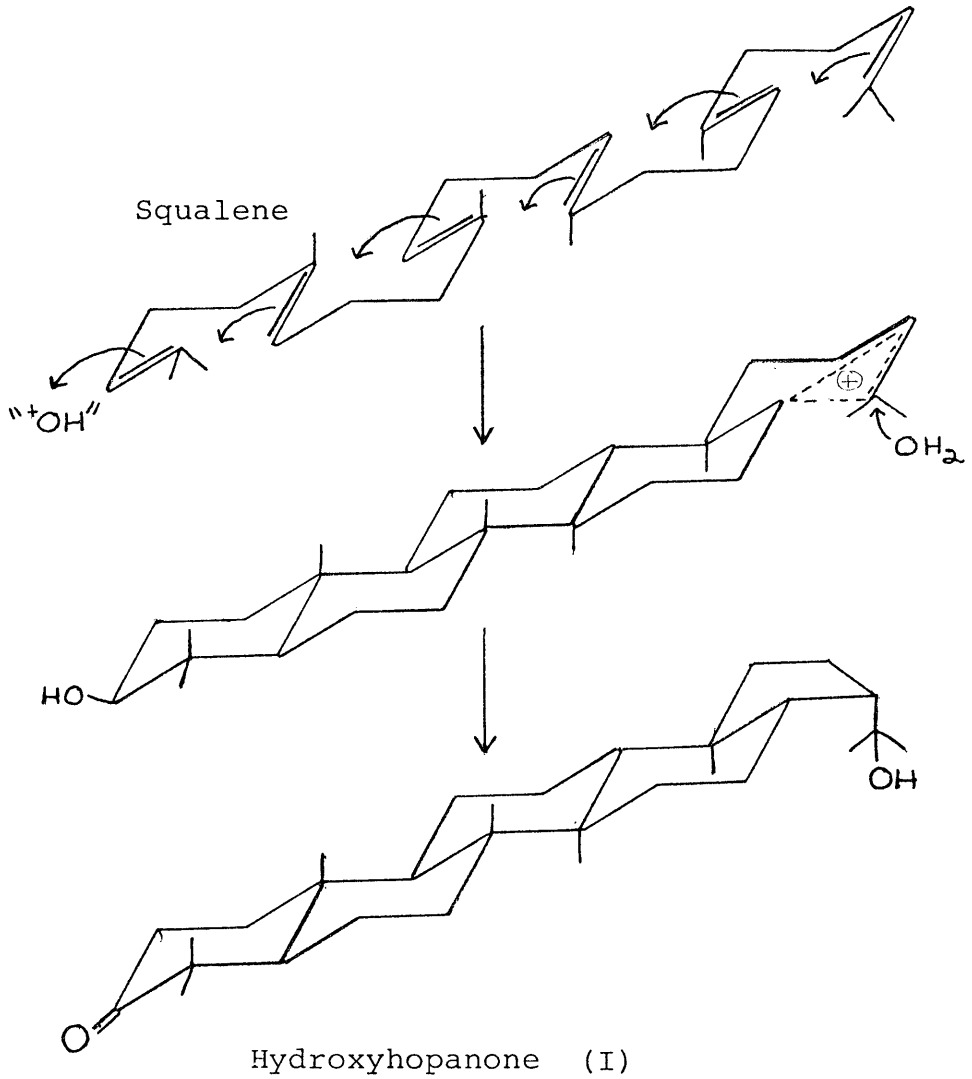
Hopenes were recently found to be constituents of Archaebacteria. Archaebacteria containing hopanes have been grown in concentrated sulfuric acid at 89°C. (Gunther Holzer, personal communication). This fact may have special meaning as the earth's early atmosphere and hydrosphere

are believed to have been highly acidic (Krauskopf, 1979, p. 533)

That hopanes occur in primitive organisms such as Archaebacter is not surprising. Hopanes are synthesized via the simplest type of squalene cyclization, all-chair cyclization (Figure 4: Richards and Hendricks, 1974).

The biochemical function of the hopanoids is believed to be that of a membrane rigidifier. Hopanoid molecules are placed between phospholipids in the phospholipid double layer (Ourisson et al., 1979) Ourisson et al. (1979) have proposed that hopanes may be the biochemical precursors to more complex cyclic isoprenoids of membranes.

It should be noted that while hopanoids with and without an oxygen functionality at C-3 are found in land plants no hopanoid with an oxygen functionality at C-3 has ever been isolated from a bacterium. This implies that a hopanoid isolated from a sediment containing an oxygen at C-3 would probably have come from a terrestrial plant source. Hopanes have been found in oil shales, coals, crude oils, oil source beds, and other recent and ancient sediments (Ensminger et al., 1974). The fact that hopanes are ubiquitous to sediments is not surprising since their biochemical precursors are found in such a wide variety of organisms. The



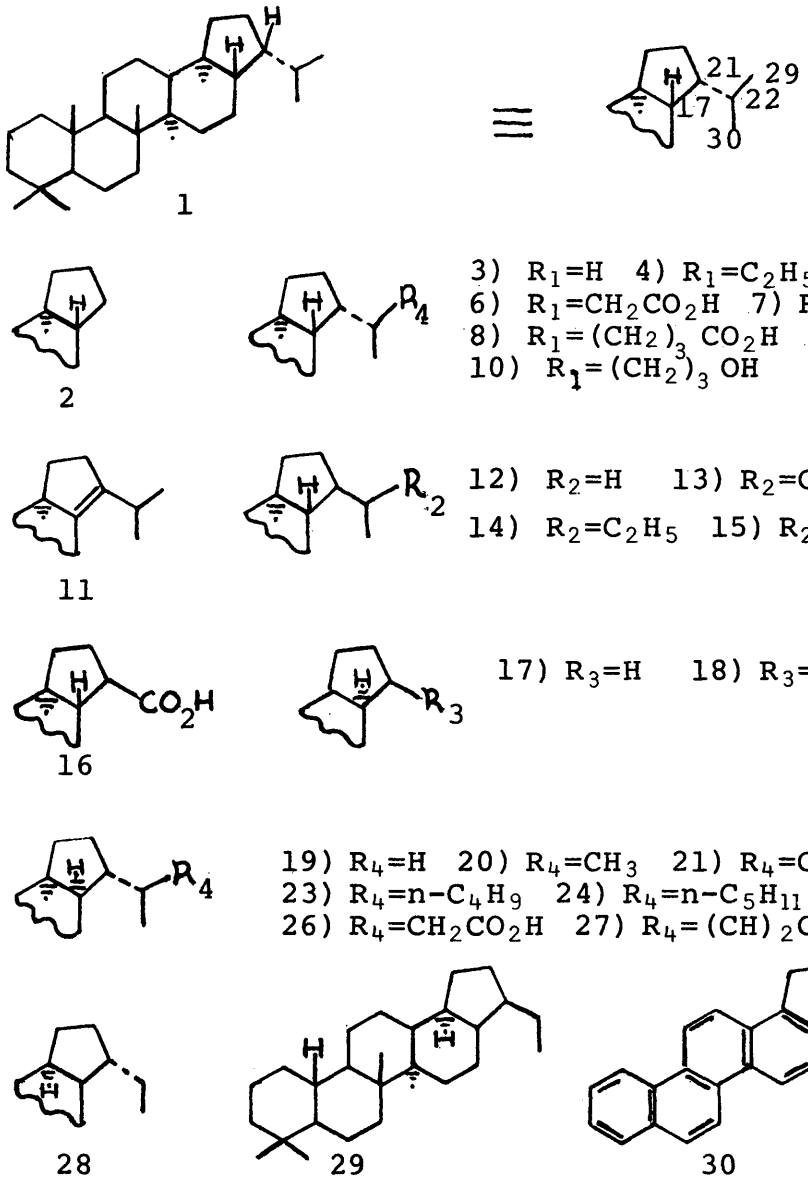
Hydroxyhopanone (I)

Simple Cyclization of All-Chair Squalene
(After Hendricks and Richards, 1964, p. 276)

Figure 4

depositional environments in which hopanes occur are extremely varied, from terrestrial to marine, pHs of 1 to 8, and temperatures of 5°C to 89°C. It must be remembered, however, that bacteria, while not major contributors to the total biomass, have the leading role in transforming organic matter prior to catagenesis. The hopanes observed after diagenesis are, therefore, most likely to be those altered or synthesized de novo by bacteria.

Examples of hopanes which have been found in recent and ancient sediments, oil shales, crude oils, and coals are shown in Figure 5. (A list of the occurrences of the hopanes and hopanoids shown in Figure 5 is given in Table 1.) Hopanoids with oxygen functionalities have also been isolated from geologic sources: Hopylacetic acid (17β(H) bishomohopanoic acid) (7, Figure 5) from the Messel oil shale, Germany; a 17β(H),21α(H) acid of the moretane series (bisnorhopanoic acid, 15, Figure 5) from the Lorraine coal field, France; and 17β(H)-homohopanoic acid (6, Figure 5) Ensminger et al., 1974; van Dorsselaer et al., 1974; and Eglinton et al., 1974) 17β(H)-bishomohopanoic acid was also isolated from the algal mats at Laguna Mormona, Baja California, along with 17β(H)-bishomohopanol (9, Figure 5) (Cardoso et al., 1975)



Hopanes and Hopanoids from Geologic Sources
Compounds are Referenced in Table 1
(Arabic numerals used for compounds found in geologic sources)

Figure 5

TABLE 1

Occurrence of hopane type compounds in sediments and crude oils (After va Dorsselaer et al, 1974, except where noted). The compounds noted are shown in Figure 5.

Location	Age	Origin	
Atlantic Ocean sediment (Mauritania)	Recent ($<10^6$ years)	Marine	6,7,8
Baltic Sea sediments	($<10^6$ years)	Marine	1-4,8,10,12,17,19-21
Gulf of Mexico sediments	Pleistocene (10^6 years)	Marine	7
Gabbon shale	Cretaceous (120×10^6 years)	Marine	2,4,7,12-14,16,17,19-21
Atlantic Ocean sediment	Creatceous (140×10^6 years)	Marine	7
Aquitaine Basin sediments (France)	Cretaceous Jurasic (150-200$\times 10^6$ years)	Marine	12-14,17,19-25
Mediano oil shale	Jurasic (180×10^6 years)	Marine	17,19-25
Lorraine coal (France)	Carboniferous (275×10^6 years)	Terrestrial	
Yallourn lignite	Miocene (25×10^6 years)	Terrestrial	12-14,16,17,19-27
Esthwaite water, Rostherne Mere & Grasmere sediments (England)	Recent (0-50 years)	Lacustrine	2,4,7,17,19-21
Messel oil shale (Germany)	Eocene (50×10^6 years)	Lacustrine	1-8,11,12,15,17-21,27,30
Bouxwiller shale	Eocene	Laucustrine	1-7,15,18
Menat shale (France)	Eocene (50×10^6 years)	Lacustrine	1-4
Stockstadt shale	Eocene (50×10^6 years)	Lacustrine	17,19-21
Green River oil shale (U.S.A.)	Eocene (50×10^6 years)	Lacustrine	19,20,21
Monterey shale[a] (U.S.A.)	M. Miocene ($\sim 10 \times 10^6$ years)	Marine	82
Norwegian Continental Shelf[b]	Jurasic	Marine	29
Crude oils			
Aquitaine Basin	Cretaceous Jurasic (150-200$\times 10^6$ years)	Marine	12-14,17,19-25
Nigeria, Libya, Iran	$>25 \times 10^6$ years	Marine	20
Russia[c]			28

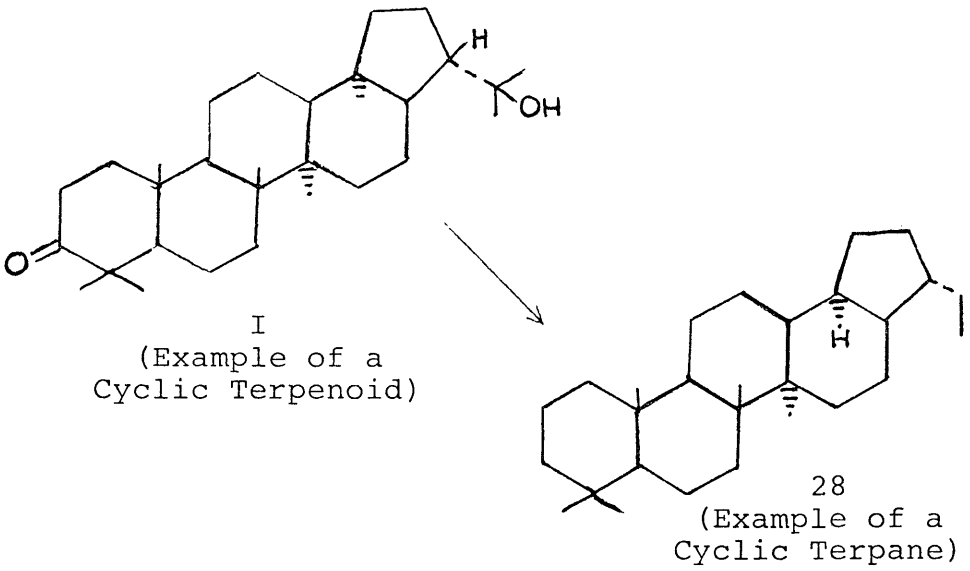
a)Seifert, 1978c
b)Bjoroy & Rullkotter, 1980
c)Seifert, personal communication

Purpose

In our experiments we have attempted to study the changes that hydroxyhopanone (I, Figure 2), a triterpenoid of terrestrial origin, undergoes when exposed to the early diagenetic conditions present in marine and fresh water depositional environments. Examples of such postulated changes are the reduction of the ketone at C-3 and the removal of the hydroxyl group from C-22, resulting in the formation of diploptene (IV, Figure 2) and other isomeric alkenes (V, Figure 2).

Subsequently, saturation of the carbon-carbon double bond and removal of the C-18 and C-30 methyl groups may result in the formation of 17α(H),18α(H),21β(H)-28 30-bisnorhopane (28, Figure 5) The net proposed transformations are shown in Figure 6. Compound 28 was recently found in the Monterey shale near Santa Barbara, California (Seifert, 1978c) It has also been found to be a major constituent of a Russian crude oil (Seifert, personal communication)

Assuming that compound 28 is derived from hydroxyhopanone, or some similar triterpenoid, it is not known how the 18α-methyl group is removed. The removal may take place via an enzymatic process. Loss of the hydroxyl group and



I
(Example of a
Cyclic Terpenoid)

28
(Example of a
Cyclic Terpane)

Net Proposed Transformation of Hydroxyhopanone (I) to 17α(H)-18α(H)-21β(H)-28, 30-bisnorhopane (28)

(Roman numerals are used for natural products, Arabic numerals are used for biological markers)

Figure 6

the C-30-methyl group may take place by either biological or abiological processes.

These experiments were designed to test the validity of the hypothesis that hydroxyhopanone may be one of the precursors to compound 28, or to other hopanes which have been found to be products of diagenesis or catagenesis in natural sediments.

EXPERIMENTAL WORK

Instrumentation:

Gas Chromatography-Mass Spectrometry and Mass Chromatography

GC-MS analyses of the samples from the incubation experiments were performed using an AEI Kratos MS-30 double focusing mass spectrometer interfaced with a Varian Model 940 gas chromatograph. The column used was a 12.5 M x 0.25 mm WCOT SE-52 glass capillary. The data system used was an AEI DS-55 software package. The instrument is located at the Branch of Oil and Gas of the United States Geological Survey, Lakewood, Colorado.

Mass chromatograms of each of the fractions from each column were made selecting specific ions known to be produced by fragmentation of the hopanes and hopanoids. For the heptane fractions the masses scanned for were 189 and 191. For the benzene and chloroform fractions the ion of mass 205 was used to search for compounds related to the starting material. In the methyl ester fractions the masses employed were 189, 205, and 235. These fragments are discussed in more detail in the Discussion section.

Infrared Spectrophotometry

IR Spectra were obtained using a Perkin Elmer Model 700 spectrophotometer. Samples were run as KBr pellets.

Electrodes

Eh electrode; platinum wire - mercury - copper wire junction in: (1) 2 mm outer diameter (O.D.) by 32.5 cm glass tubing, and (2) 2 mm O.D. by 13 cm glass tubing. One inch of platinum wire, 16 gauge was exposed at the bottom of each electrode.

Reference electrode; 3M KCl silver - silver chloride electrode; Micro-Reference Electrode - MI 409; 2.5 mm O.D. by 14.4 cm total length. Supplied by Microelectrodes Inc., Londonderry, New Hampshire.

pH electrode; Miniature Glass Electrode - MI 405; 2 mm by 14.6 cm total length. Supplied by Microelectrodes Inc.

pH meter; the meter used for pH and Eh measurements was a Corning Model 12 research pH meter.

Solvents and Glassware

All solvents were reagent grade and were distilled prior to use by rotary vacuum distillation using a Buchler Rotary Flash Evaporator. All glassware was precleaned in either a KOH-diethylene glycol bath or a chromic acid bath for 24 hours.

Isolation of Hydroxyhopanone from Dammar Resin

The procedure for the isolation of hydroxyhopanone is a slightly modified version of that given by Dunstan et al., 1957. Approximately 40 g of finely ground Dammar resin* (Sigma) was packed into a 33 x 94 mm cellulose extraction thimble, and Soxhlet-extracted with 125 ml of methanol for four hours. Four extracts were then combined in a 1000 ml round bottom flask and the methanol removed under reduced pressure using a Buchler flash evaporator. Roughly 100 g of residue remained in the flask. The residue was dissolved in 450 ml of ether. The Dammar acids were removed by extraction with three 80 ml portions of a 5% KOH aqueous solution. The ethereal solution was extracted with deionized water to remove the base. If an emulsion persisted a saturated salt solution was added to the separatory funnel to break the emulsion.

The ethereal solution was then transferred to a 600 ml beaker and $MgSO_4$ was added as drying agent. The beaker was covered with plastic wrap to prevent evaporation and placed in a refrigerator at 5°C until the solution became clear. The $MgSO_4$ was removed by vacuum filtration and washed with

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*Dammar resin is the crude extract of trees of the Dipterocarpaceae family of Angiosperms found in Southeast Asia.

ether. The ether filtrate was then placed in a clean 600 ml beaker and the volume adjusted to 200-300 ml. Again the beaker was covered and placed in a freezer at 0°C (or below) for one week or longer. A solid precipitated, and the ether was decanted. The solid was washed with cold ether and redissolved in 500 ml of acetone. The solvent was then evaporated using a hot plate-magnetic stirrer combination. When the volume had been reduced to 200 ml the hot plate and stirrer were turned off and the solution was allowed to cool slowly. As the acetone continued to evaporate crystals began to appear as needles. After crystal growth appeared to have stopped, the solution was refrigerated and the crystals were collected by filtration. Each batch yielded approximately 300 mg of material. The twenty batches gave a total of 6 g of material.

A thin layer chromatogram of each batch showed that the material was made up of two components. These components were then resolved by liquid column chromatography as described below.

One gram of the material isolated from Dammar resin was dissolved in $CHCl_3$ and adsorbed onto 20 g of silica gel (100-200 mesh) Solvent was removed by rotary evaporation. The silica gel with the material adsorbed to it was then added to a column, 3 cm x 30 cm, containing 100-200 mesh

silica gel packed as a slurry in heptane. The column was then eluted with one liter each of the following: 50% (v/v) heptane-benzene, benzene, 50% (v/v) benzene-chloroform, and chloroform. Fractions were collected every 200 ml, except the first two fractions, which contained 400 ml each. Eighteen fractions were collected from each experiment. The procedure was repeated six times.

Thin-layer chromatography and IR were used to monitor the columns. Hydroxyhopanone eluted in the benzene-chloroform fractions. These fractions from each column were then combined to give 5.28 g of a mixture of largely two isomers of hydroxyhopanone. (See results: Analysis of the material isolated from Dammar resin and purified by column chromatography.) It was this mixture which was used in the incubation experiments. The flow chart for the isolation of hydroxyhopanone is shown in Figure 7.

Scheme for the Isolation of Hydroxyhopanone

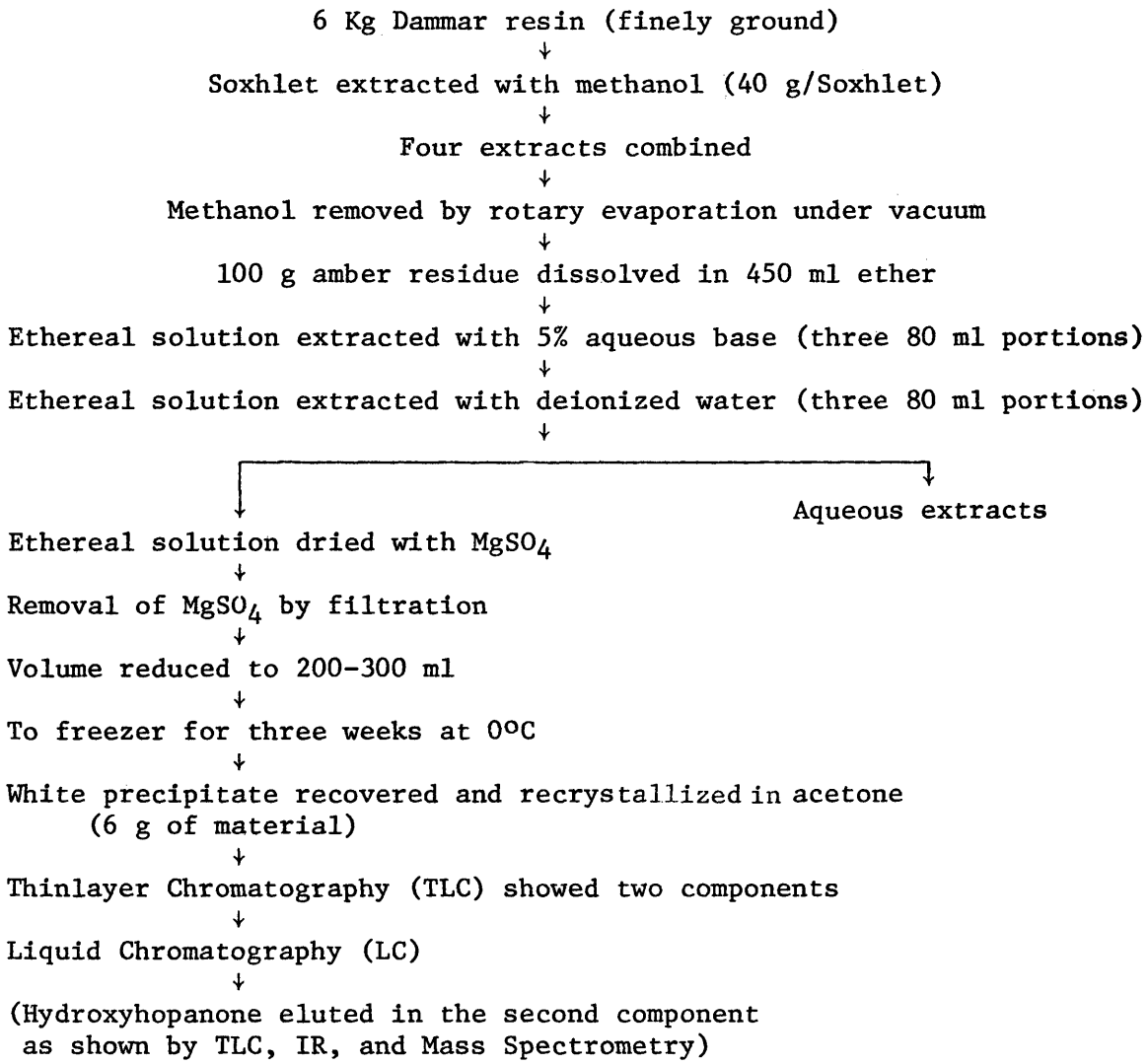


Figure 7

Thin Layer Chromatography

All thin-layer chromatography was performed using silica gel (Silicar-7G, Malinkroft) as the adsorbent. The thin layer plates were made by dipping microscope slides into a slurry of chloroform and adsorbent. The plates were spotted with sample in the usual manner, eluted in chloroform, and developed in iodine.

Anoxic Column Setup

The mineral matrix for the anaerobic columns consisted of an anoxic marine sediment from off the western coast of Mexico. The location of the sample site was 23°35'N. Lat. and 107°26' N. Long. The sample was taken from the sediment surface by box core in 530 m of water depth. The core was supplied by Andrew Soutar of Scripps Institution of Oceanography, La Jolla, California.

The core was maintained at 0°C or less while at Scripps, was refrigerated for one week at 5°C here at Mines, and was maintained for one year at dry ice temperatures until used. The sediment had been allowed to thaw twice during its year at CSM: once in order to set up R. Parker's initial experiments, and a second time when a fresh supply of dry ice failed to arrive.

Glass columns, 1 inch x 12 inches, were sealed on one end by typical glass-blowing technique. The columns were

then washed and sterilized by flaming with 70% ethanol in water. Thirty-five ml of the appropriate sea water solution was added to the columns. (See Table 2 for descriptions of the columns and the solutions which they contained.) Next, to those columns which were to contain hydroxyhopanone, the mixture (approximately 300 mg) was injected as a slurry in dimethylformamide (DMF) using a ground glass 5 cc syringe with a 4-inch (17-gauge) needle. The marine sediment was added using a large spatula. One hundred grams of the sediment was delivered through the water to the bottom of the columns by ultrasonic vibration in an ultrasonic bath. The columns were then stoppered with teflon-wrapped silicone stoppers. The stoppers were epoxied into the column and the seal was wrapped with teflon tape. Each teflon stopper had two 1/4-inch glass "T's" running through it for sampling, performing bacterial plate counts, and introducing microelectrodes to make Eh and pH measurements. The system was also designed in this way so that it could be purged with nitrogen. (See Figure 8.)

After the columns were set up they were flushed with prepurified nitrogen to remove air from the gas cap between the water and the stopper. While the system was purged the "T's" were closed with eye dropper bulbs. However, these seals were not complete and the system would depressurize.

TABLE 2

Anoxic Columns

Column Code	Column Type	Contents*	Incubation Time (Days)
An1	"Living"	Solution 1	60
An2	"Living"	Solution 2	60
An3	"Living"	Hydroxyhopanone + Solution 2	60
An4	"Living"	Same as An3	60
An5	Poison Control Analogue of An1	Solution 4	60
An6	Poison Control Analogue of An2	Solution 3	60
An7	Poison Control Analogue of An4	Solution 3	60
BanZ	Blank Zero-time	Solution 1	60
HanZ	Hydroxyhopanone Zero-time	Hydroxyhopanone + Solution 1	0

*All columns contained 100g of wet sediment and 35 ml of an artificial sea water solution (Instant Ocean, Aquamarine Systems) Columns An2, An3, An4, An6, and An7 contained 10 ml of dimethylformamide.

Solutions:
1 - Sea Water only.

2 - Sea Water and Nutrients; 0.7g glucose and 0.7g yeast extract dissolved in 250 ml of sea water.

3 - Sea Water, Nutrients, and Bactericide; 0.7g glucose, 0.7g yeast extract, and 1.2g HgCl2 dissolved in 250 ml sea water.

4 - Sea Water and Bactericide; 1.2g $HgCl_2$ dissolved in 250 ml of sea water.

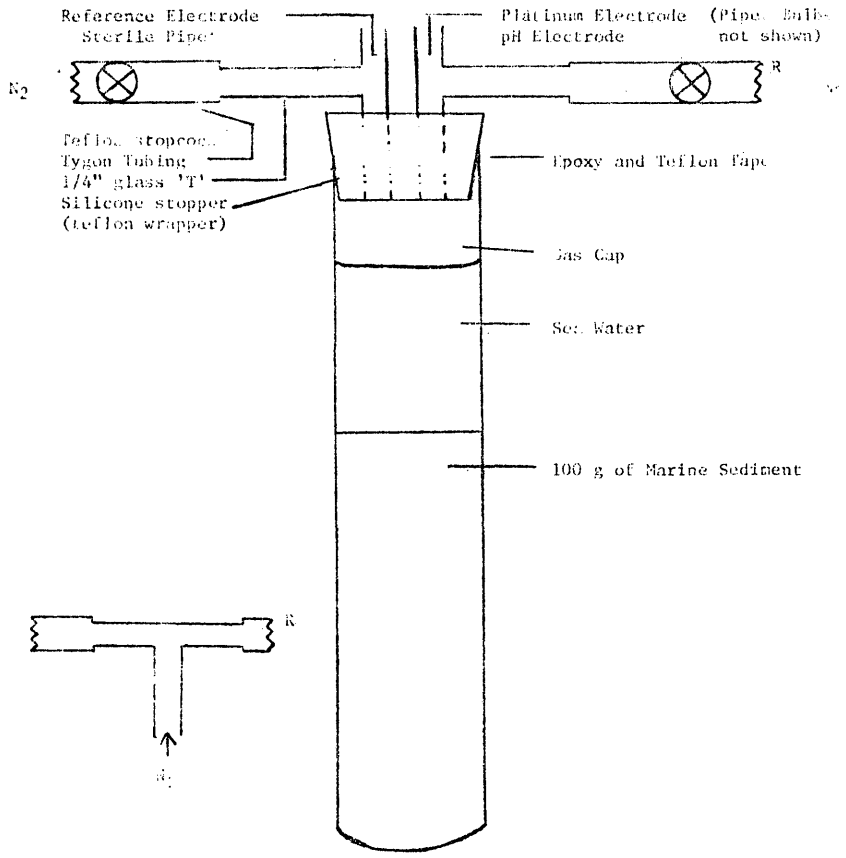


Diagram of an Anoxic Column

Figure 8

The columns which did not contain hydroxyhopanone were set up in the same manner, but did not include the second step. In addition, two zero time columns were set up: a blank and a column containing the compound. These were frozen and then disassembled and extracted soon afterward. This was done to serve as a check on the efficiency of the extraction so that it would be known what to expect from the remaining columns.

Seven columns were incubated: Columns An1 through An7 of Table 2. The incubation was carried out in a 50-gallon water bath, in order to moderate the daily fluctuation in temperature. The incubation period was 60 days. The temperature of the water bath varied between 19°C and 26°C. A more geologically-realistic temperature of 4°C was not used because the microbial growth would be retarded and therefore any alterations which might take place would have taken longer to occur.

Table 2 shows the columns which were set up. Columns An5, An6, and An7 were poisoned controls, using $HgCl_2$ as the poison. These controls were set up in order to help distinguish between any abiological alteration products and the biological alteration products of the columns containing hydroxyhopanone.

Once all the columns had been placed in the water bath the bath was wrapped with aluminum foil to minimize the amount of light reaching the sediment in order to inhibit photosynthesis.

Oxic Column Setup

Aerobic stream sediment was collected with shovel and bucket from the North Fork of Middle Boulder Creek by R. Parker, Colorado School of Mines. One gallon of water was also collected. The sediment and water were stored at 5°C for one year.

The glass columns used in this set of experiments were made in the same manner as those used in the anoxic experiments. However, since these columns were to be maintained aerobically, they were set up such that air could be bubbled through the sediment, as shown in Figure 9.

The aerobic stream sediment was added to the columns while air was running through the air line. After half of the sediment had been added, half of the mixture of the two isomers of hydroxyhopanone was added to the appropriate columns (columns A3, A4, and A7) The remainder of the mixture was added after all of the sediment was added (80g). The mixture (300 mg of a mixture of largely two isomers, 40-45% of which was hydroxyhopanone) was added to the columns as a slurry in dimethylformamide and injected with a

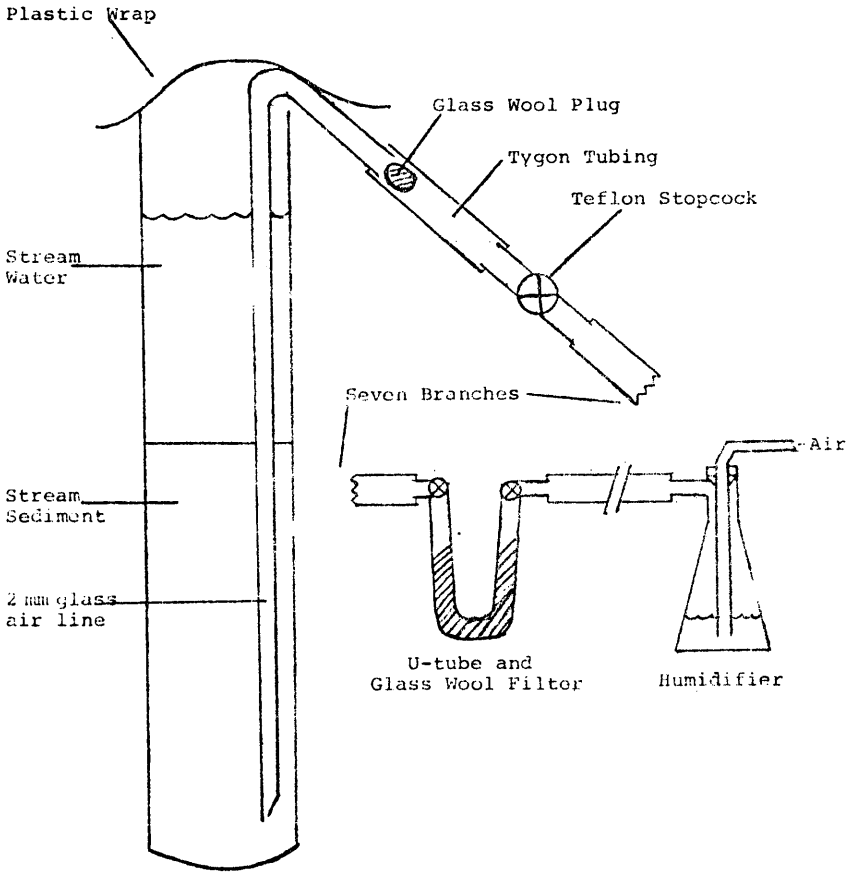


Diagram of an Oxic Column

Figure 9

5 cc ground glass syringe with a 4-inch needle attached. After all the sediment or mixture had been added, 35 ml aliquots of stream water solutions were then added to the sediment (Table 3).

Seven columns were incubated in the same manner as the anoxic columns (Table 3) The incubation period for column A3 was 30 days, and 60 days for the other columns. Two zero-time columns were set up (BAZ and HAZ) as in the anoxic set. Columns A5, A6, and A7 were poisoned controls used to distinguish abiological alteration products from biological alteration products.

After the columns were in place in the water bath each was covered loosely with plastic wrap (Dow) to prevent evaporation. Evaporation did occur, however, and the columns were replenished with their corresponding solution.

Eh Measurements

Prior to the measurements the anoxic columns were purged with prepurified nitrogen. The measurements were made by lowering the reference and platinum electrodes into the columns via the glass "T's" while maintaining the nitrogen purge. Readings were taken with the platinum electrode in three positions: (1) 1-2 cm above the water-sediment surface; (2) 1-2 cm below the water-sediment interface; and (3) at the bottom of the column. Readings were taken

TABLE 3

Oxic Columns

Column Code	Column Type	Contents*	Incubation Time (Days)
A1	"Living"	Solution 1A	60
A2	"Living"	Solution 2A	60
A3	"Living"	Hydroxyhopanone + Solution 2A	30
A4	"Living"	Same as A3	60
A5	Poison Control Analogue of An1	Solution 4A	60
A6	Poison Control Analog of An2	Solution 3A	60
A7	Poison Control Analogue of An3 and An4	Hydroxyhopanone + Solution 3A	60
BAZ	Blank Zero-time	Solution 1A	0
HAZ	Hydroxyhopanone Zero-time	Hydroxyhopanone + Solution 1A	0

*All columns contained 80g of stream sediment and 35 ml of a stream water solution. Columns A2, A3, A4, A6, and A7 contained 10 ml of dimethylformamide.

Solutions:

1A - Stream Water only.

2A - Stream Water and Nutrients: 0.7g glucose and 0.7g yeast extract dissolved in 250 ml of stream water.

3A - Stream Water, Nutrients, and Bactericide; 0.7g glucose, 0.7g yeast extract, and 1.2g $HgCl_2$ dissolved in 250 ml of stream water.

4A - Stream Water and Bactericide; 1.2g $HgCl_2$ dissolved in 250 ml of stream water.

at the bottom of the column beginning on the 20th day of incubation. The reference electrode was never lowered into the the sediment. The platinum wire was polished with emery cloth between measurements in each column. The electrodes were standardized against ZoBell's solution between column measurements. Eh measurements were made solely on the anoxic columns.

pH Measurements

The pH of the sea water in the anoxic columns was measured in the same manner as the Eh, but was not done below the sediment-water interface. The electrodes were standardized with a pH 9 buffer. pH of the aerobic columns was obtained by inserting the electrode to within 1-2 cm above the sediment surface. The plastic cover was removed and the air to each individual column was turned off prior to the measurements.

Sampling and Bacterial Counts

Water samples were taken in order to perform bacterial plate counts. Samples were taken from the anoxic columns by inserting a sterile 1 ml pipet through one of the two "T's" with the other "T" closed off, and with the nitrogen flushing the system until the pipet reached the gas-water interface; then the nitrogen was turned off. The purge was begun again after the pipet with sample had been extracted.

Samples taken from the sediment-water interface were usually less than 0.5 ml. Beginning on the 30th day samples were also taken from 3 to 4 cm below the water-sediment interface. Aerobic stream water samples were removed by inserting a sterile 1 ml pipet to the water-sediment interface with the air turned off.

Bacterial growth was monitored by enumerating the colony-forming units per milliliter (CFU/ml). The colony-forming units were counted from spread plates. The plates were made in the following manner: a 0.1 ml sample was diluted 100, 1000, and 10,000 times (or higher) and 0.1 ml of each of the three (or more) dilutions was plated. The whole procedure was performed using aseptic technique.

The number of colony-forming units per milliliter was found by the following formula:

$$\text{CFU/ml} = \text{Colonies per plate} \times \text{dilution factor} \times \frac{10 \text{ tenth ml}}{\text{ml}}$$

Aerobic plates were made using sterile deionized water for the dilutions and Standard Methods Agar (Pasco Labs, Wheatridge CO). The aerobic plates were incubated at atmospheric pressure and 28-29^{o}C.

Anaerobic plates were made with sterile sea water (Instant Ocean, Aquamarine Systems, Eastlake, Ohio) and ZoBell's Marine Agar (ZoBell, 1946a, made by Pasco Labs). The

anaerobic plates were incubated in an autoclave which was evacuated and repressurized to 5 psi with hydrogen. The autoclave was left at room temperature, 22°C-25°C. All plates were incubated for 72 hours and then counted. They were reincubated and counted again four days later. With each set of plates, aerobic or anaerobic, a blank control plate was added to serve as a check for contamination.

Column Extraction

At the end of the incubation period the columns were removed from the water baths and frozen. They were then cut into three (oxic) or four (anoxic) sections, one of water, and two or three of sediment, using a circular diamond saw. The outside of each sediment section was washed with methanol, and then placed into a Soxhlet thimble (94 x 33 mm single thickness, Whatman). The sediment sections were allowed to thaw and drain into a beaker, while being rinsed with methanol (reagent, MCB) to remove the interstitial water. The sediment sections were then extracted in a Soxhlet apparatus with 250 ml of chloroform for 16 hours.

The methanol-water solutions were filtered by vacuum filtration (Buchner funnel with Whatman #5 paper) and the methanol removed by rotary-vacuum evaporation. These sections were acidified to pH 1-2 and extracted with methylene chloride (4 x 35 ml).

After removal of some of the chloroform from the sediment extracts by rotary-vacuum evaporation, the methylene chloride and chloroform extracts were combined and filtered as previously described. The combined extracts were again vacuum-evaporated. When approximately 30 ml of extract remained it was transferred to a preweighed 35 ml vial and dried under nitrogen at 40°-45°C. The weight of the dried extracts were then obtained (Appendix B)

Sample Preparation: Anoxic Columns

The extracts (dried and weighed) were dissolved in 30 ml of chloroform (MCB) A 10 ml aliquot was removed and dispensed into a 100 ml round bottom flask, and the solvent was removed by rotary evaporation. The residue was then saponified in 50 ml of a 5% (W/W) KOH-methanol solution at 40-45°C for 8 hours (5 ml of benzene was added to completely dissolve the sample) The reaction mixture was stirred using a teflon magnetic stir bar.

After saponification, the methanol was removed by rotary evaporation and the sample was redissolved in 50 ml of deionized water. The basic solution was extracted with benzene (6 x 25 ml) to remove the nonsaponifiables. The aqueous phase was then acidified to pH 1-2 and extracted with n-heptane (MCB: 4 x 25 ml)

The benzene was removed from the nonsaponifiables by rotary-vacuum evaporation. The residue was then redissolved in 0.5 ml of chloroform and eluted on an activated silica acid (100-200 mesh) column (Unisil, Clarkson Chemical Co., Williamsburg, Virginia) The column was 35 cm x 1.2 cm and was packed as a slurry in n-heptane. All columns were eluted sequentially with 200 ml each of the following solvents: heptane, benzene, chloroform, and methanol. Four fractions were collected corresponding to each solvent. The solvents were removed as previously described and the samples dried under nitrogen at 35°C. The weight of each fraction was then obtained (Appendix B)

The n-heptane was removed from the saponifiable fractions by vacuum rotary evaporation. The carboxylic acids of the residue were then methyl-esterified by dissolving the residue in 1-2 ml of $BF_3(CH_3OH)_2$ and heating the reaction mixture at 100°C for 5 minutes in a diethylene glycol bath. The reaction mixture was then quenched with water (25 ml) and the methyl esters extracted with n-heptane (2 x 5 ml). As with the nonsaponifiables, a dry weight of the esterified fractions was obtained (Appendix B)

Sample Preparation: Oxic Columns

The saponification and subsequent separation of saponifiables from nonsaponifiables of the aerobic samples were

handled in the same manner as for the anoxic samples. However, since the blanks were lean with respect to organic content the whole sample was saponified.

The nonsaponifiables were also separated into four fractions via liquid chromatography. The columns were 10 cm x 1 cm silicic acid 100-200 mesh (Unisil, Clarkson Chemical Co.) The columns were packed as a slurry in n-heptane. Sample introduction was performed as previously described. Elution was carried out in the following manner: 60 ml n-heptane, 60 ml benzene, 80 ml $CHCl_3$, and 60 ml MeOH. Four fractions were collected per column. The fractions were then treated in exactly the same manner as were the anaerobic fractions.

Esterification of the carboxylic acids in the saponifiable fraction was carried out in exactly the same way as it was with the anaerobic saponifiables.

Prior to injection into the GC-MS the dried samples were dissolved in a suitable solvent. The heptane fractions were dissolved in heptane, and the benzene, chloroform, and methyl esters were dissolved in chloroform. The methanol fractions were not analyzed.

A flow chart describing how the sediment columns were handled after incubation is shown in Figure 10.

Scheme for Sample Separation

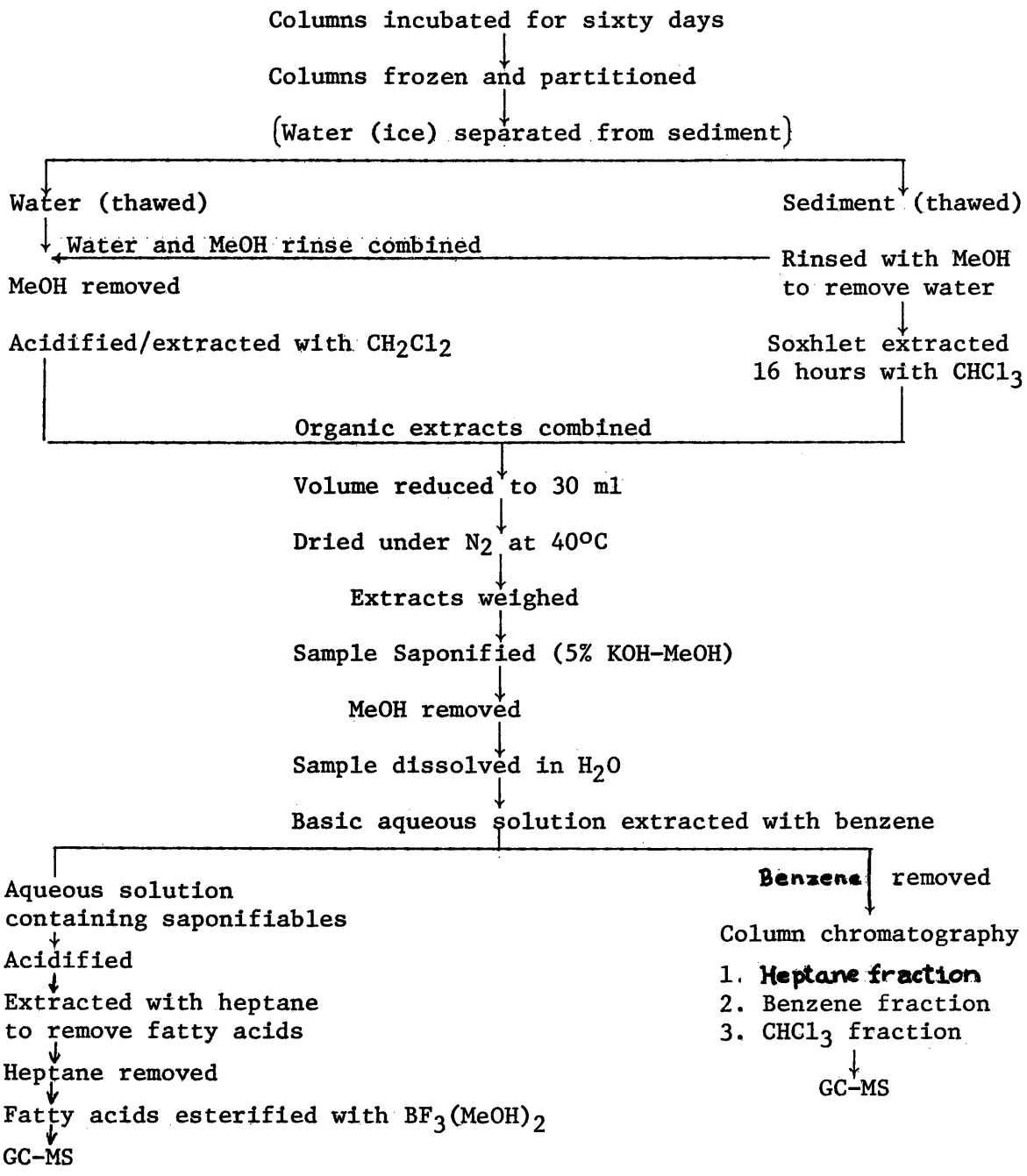
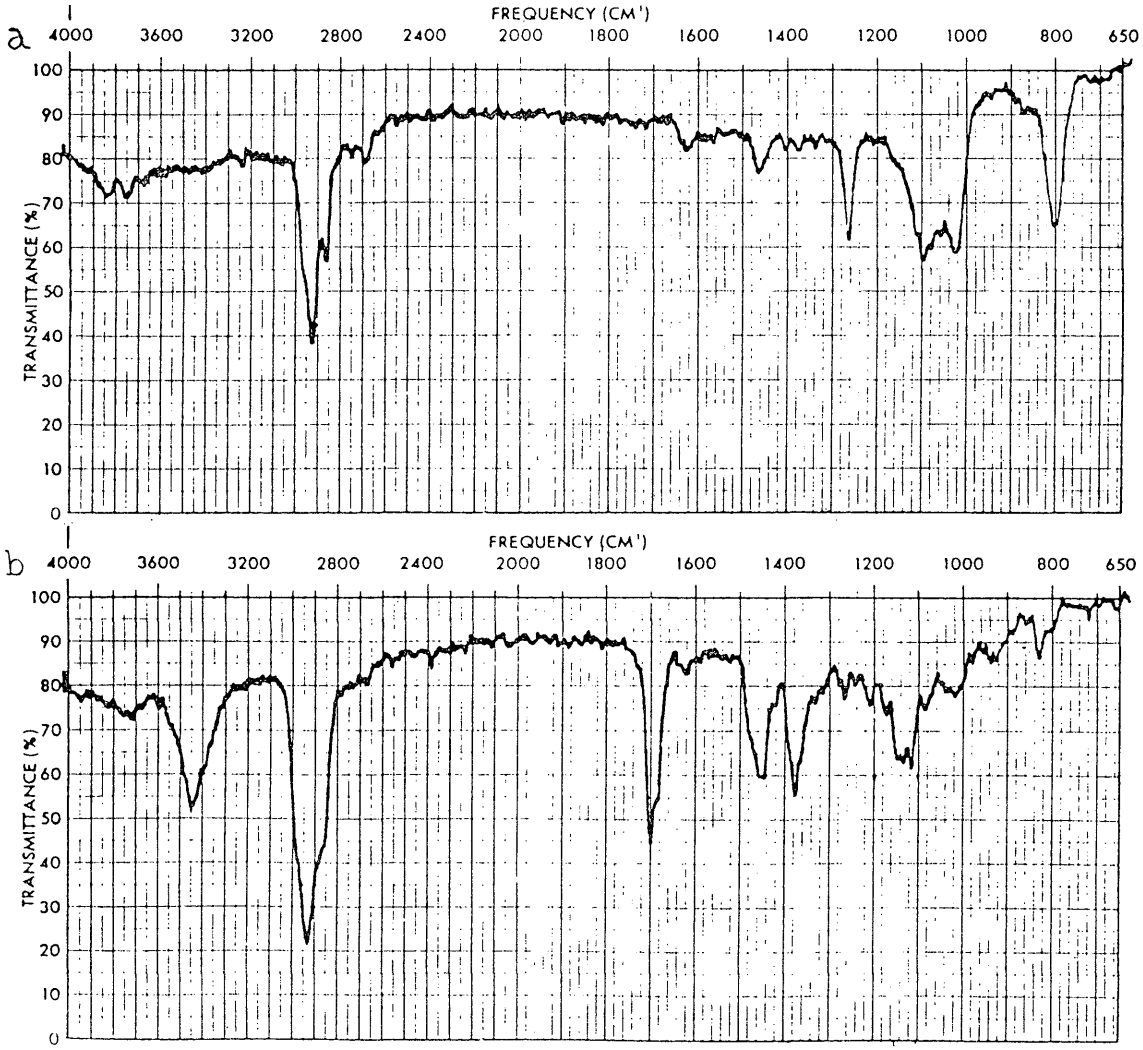


Figure 10

RESULTS

Analysis of the Material Isolated from Dammar Resin and Purified by Column Chromatography

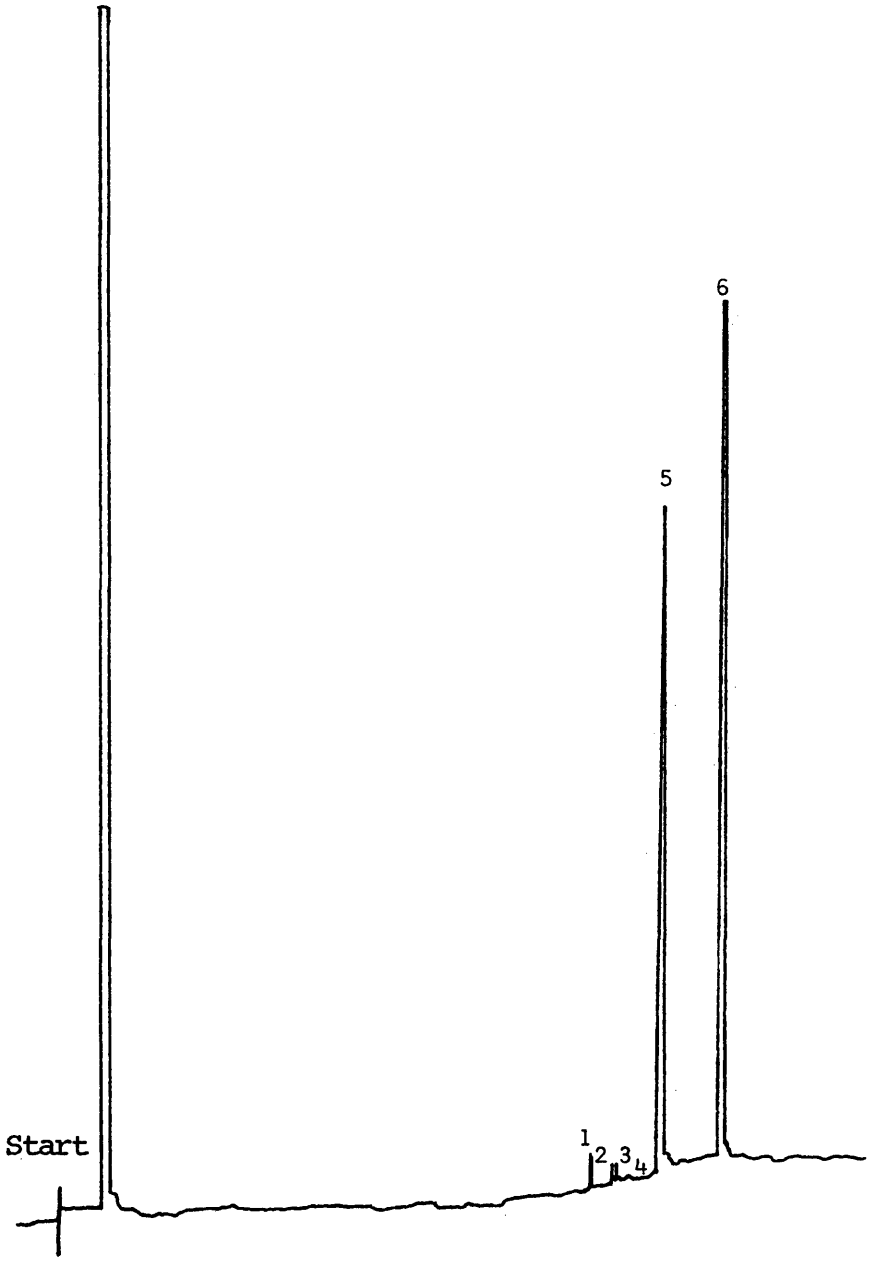
Infrared spectra of the two components of the initial product isolated from Dammar resin and separated by column chromatography are shown in Figures 11a and 11b. Figure 12 is a gas chromatogram of the material believed to be hydroxyhopanone. The IR of this material is given in Figure 11b. Figure 13 is the total ion current of a GC-MS experiment performed on the same material analyzed by GC (Figure 12). Mass spectra of the two major components (peaks 5 and 6) shown in Figures 12 and 13 are given in Figures 14a and 15. Figure 14b is a mass spectrum of authentic hop-22(29)-en-3-one obtained from a spectral library search. Figure 16 is a mass spectrum of the mixture analyzed by direct probe insertion into the mass spectrometer.



a - IR of the first component to elute during column chromatography of the material extracted from Dammar resin.

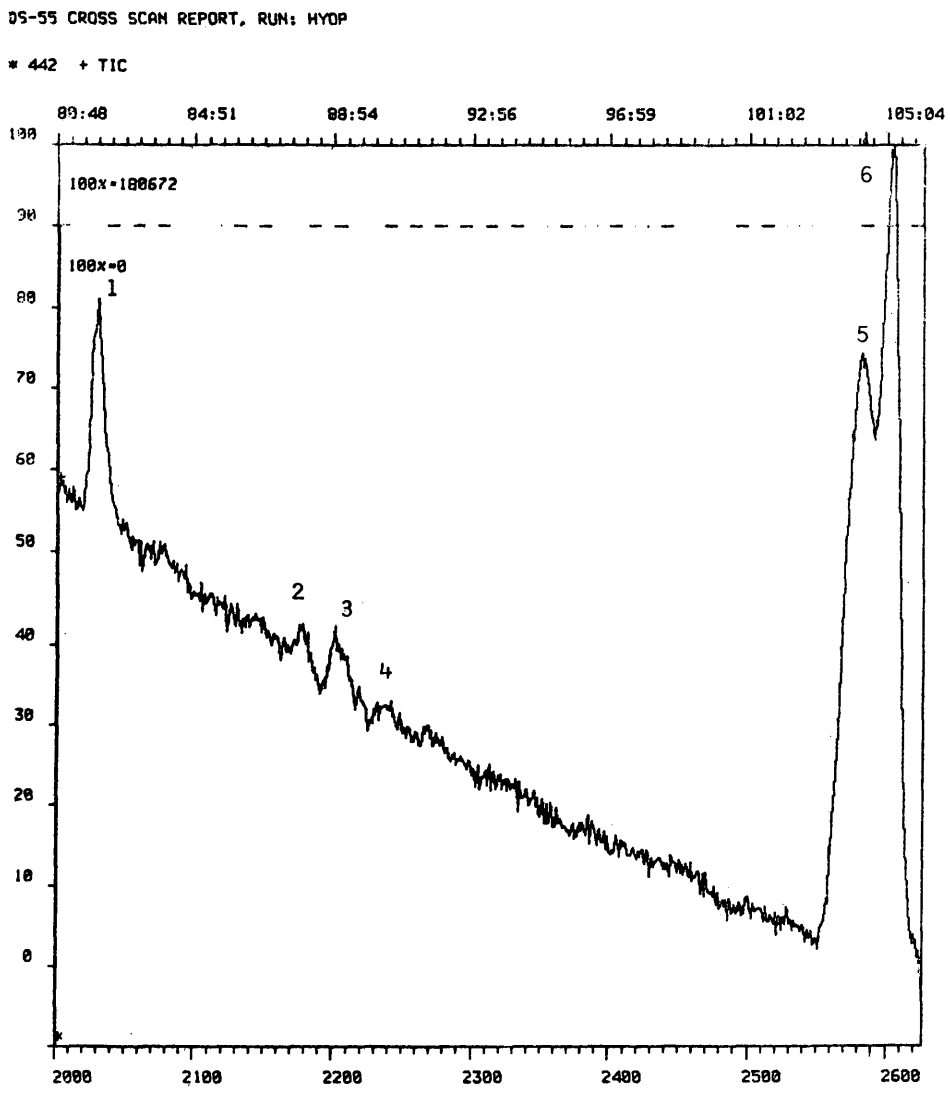
b - IR of the second component: Note the broad hydrogen bonded OH stretch (3600-3300 cm^{-1}) and the ketone response at 1700 cm^{-1} This fraction contained hydroxyhopanone.

Figure 11



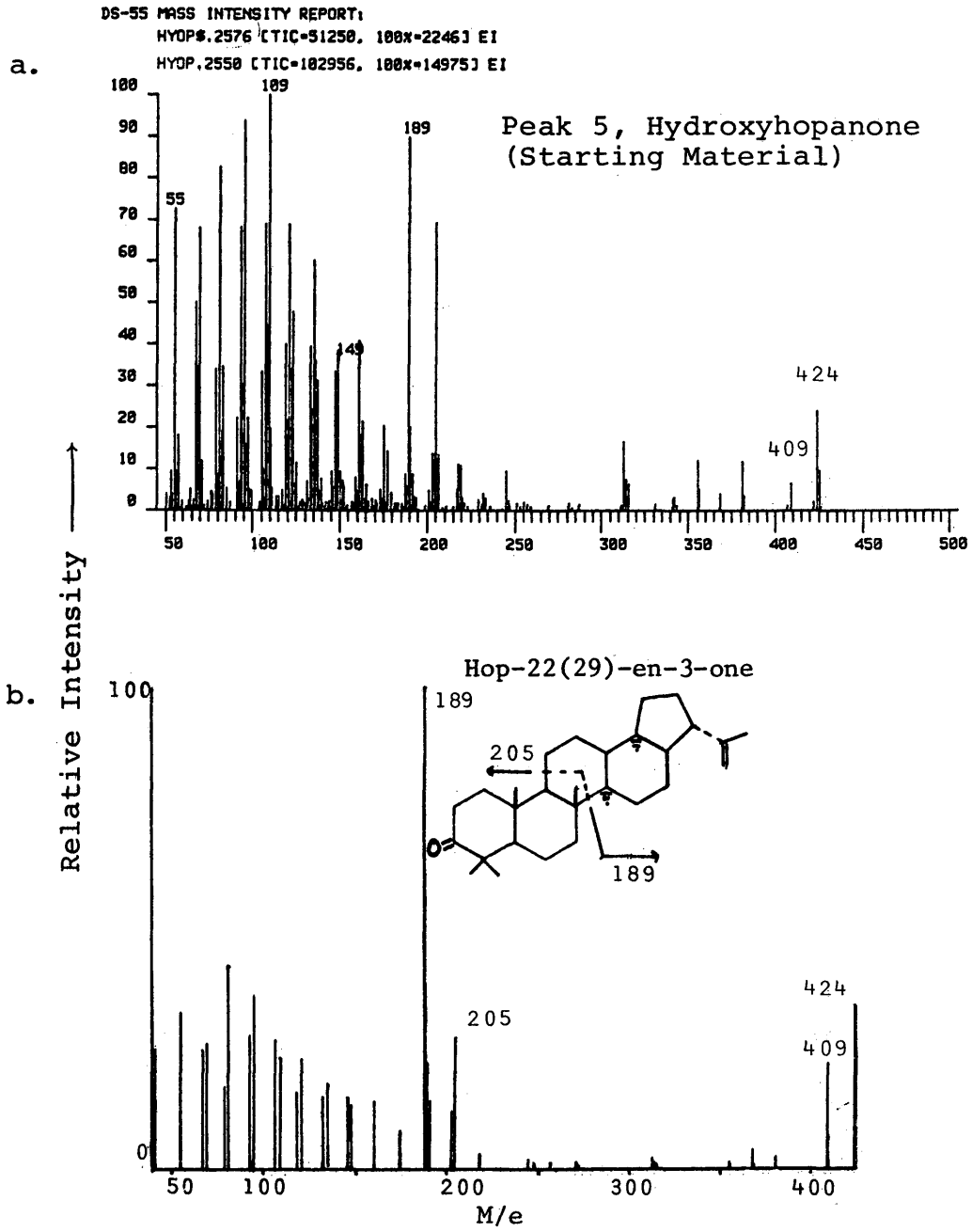
Gas chromatogram of the material isolated from Dammar resin and purified by LC. GC parameters - 30 M x 0.25 mm SE-52 glass capillary, 200-300°C at 5°C/min, 2.5 ml/min (chart 0.5 cm/min) Courtesy of Dr. D. Miiller, Phillips Petroleum.

Figure 12



Total Ion Current (TIC) of same material as in Figure 12. GC parameters: 30 m x 0.25 mm SE-54 glass capillary, 200-300°C 2 ml He/min. Top: DS-55-data system used, scanned for all masses (TIC) and mass 442 (molecular ion). Molecular ion was not detected but rarely is for alcohols. X-axis: top, time in mins; bottom, scann No. Y-axis: Relative intensity in percent normalized to largest peak(6) Courtesy Dr. D. Miiller, Phillips Petroleum.

Figure 13



Mass spectrum of peak 5 (a) (Fig. 13). This component is believed to be hydroxyhopanone by comparison with the mass spectrum of hop-22(29)-en-3-one(b) obtained by mass spectral library search.

Figure 14

Figure 15

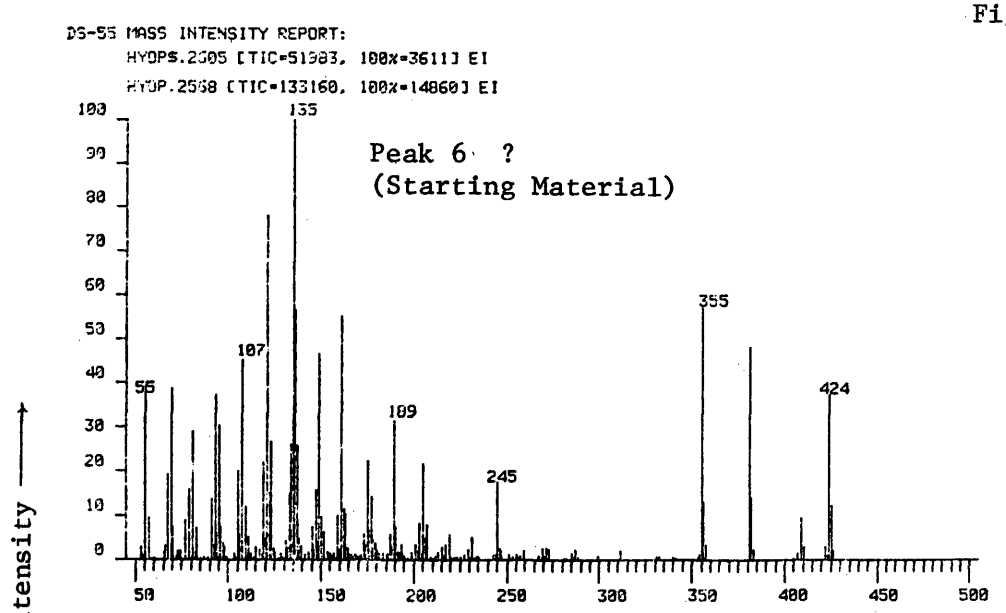
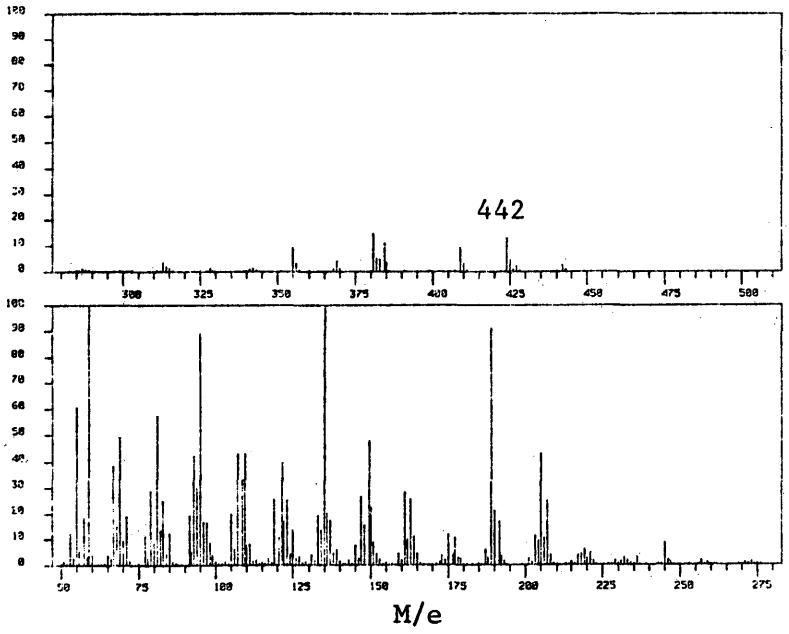


Figure 16



15. Mass spectrum of peak 6 of the starting material. This component is an isomer of hydroxyhopanone but its structure is unknown.
16. Direct probe mass spectrum of the material isolated from Dammar Resin and purified by L.C. Note the molecular ion at M/e 442. This analysis was performed at the U.S.G.S. Denver Federal Center, Branch of Oil and Gas.

Anaerobic Experiments: Bacterial Growth, Eh and pH

Bacterial growth curves for the anaerobic experiments are given in Figures 17a through 18c. The growth curves represent the multiplication of bacteria occurring just above the water-sediment interface and 3-4 cm below it. Bacterial growth below the water-sediment interface was checked only during the last 30 days of the experiment. The data are organized according to the contents of the incubated columns (see Table 2). Column An1 (circles and solid line) is contrasted with its poison control analogue, column An5 in Figures 17a and 18a. The data obtained from the measurements made on columns An2 and An6 are plotted together in Figures 17b and 18b. Since columns An3 and An4 had identical contents, the data describing these columns are plotted together, along with those of their poison control analogue, column An7. Where possible, this manner of organization is maintained in the remainder of the text.

In Figure 19 the pH of the water in each of the anaerobic columns is plotted against time. These plots are organized in the same manner as are the graphs of bacterial growth versus time.

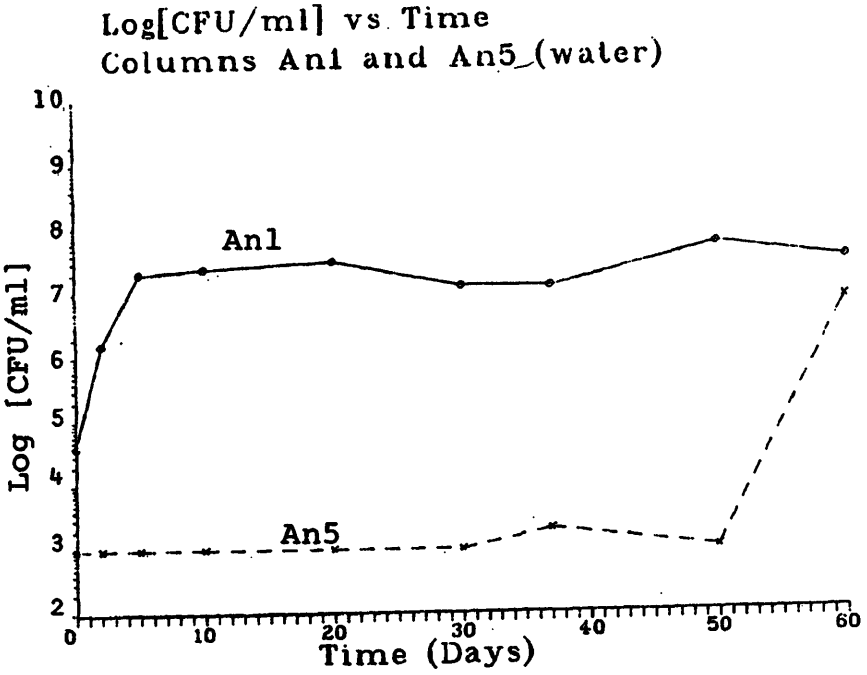
Figures 20a through 20g show the E_h of the water just above the water-sediment interface, the uppermost 3 to 4 cm

of sediment, and the bottom of the sediment. The Eh data from each column are plotted individually.

Aerobic Experiments: Bacterial Growth and pH

Bacterial growth curves for the aerobic incubation experiments are shown in Figures 21a through 21c. The plots are organized in the same manner as the anaerobic growth curves. The data from column A1 are plotted with the data from its poisoned control analogue, column A5 in Figure 21a. The same is true for the data from columns A2 and A6 of Figure 21b. In Figure 21c the data from columns A3 and A4 are plotted together along with the data from the poisoned column A7. Columns A3 and A4 had identical contents; however, column A3 was incubated for 30 days instead of 60. Samples were not taken from column A7 during the last 20 days because of evaporation.

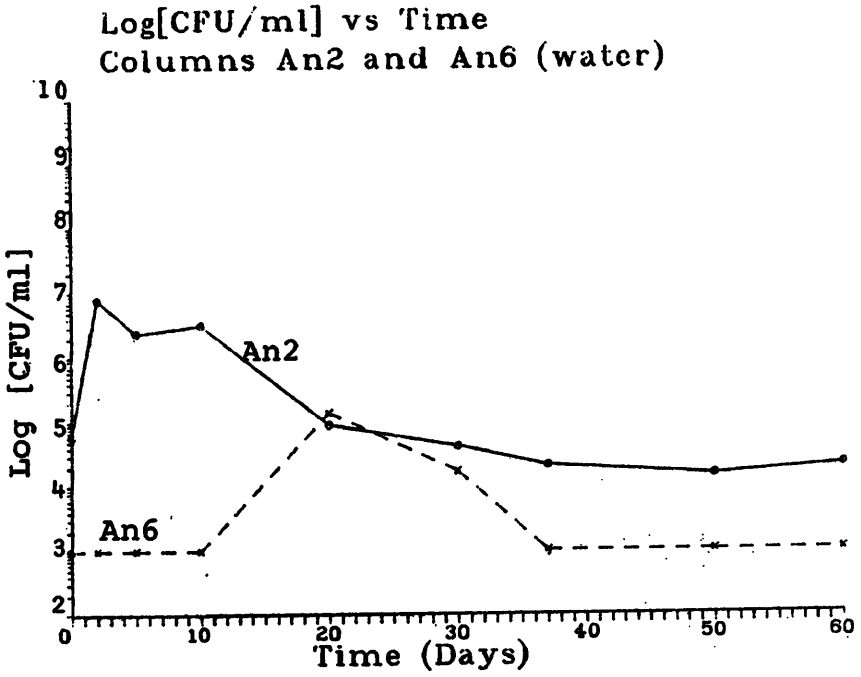
Plots of pH versus time for the aerobic waters are shown in Figure 22. The plots are organized in the same manner as are the growth curves.



Relative growth of bacteria above the water-sediment interface in columns An 1 and An 5.

CFU = Colony Forming Units

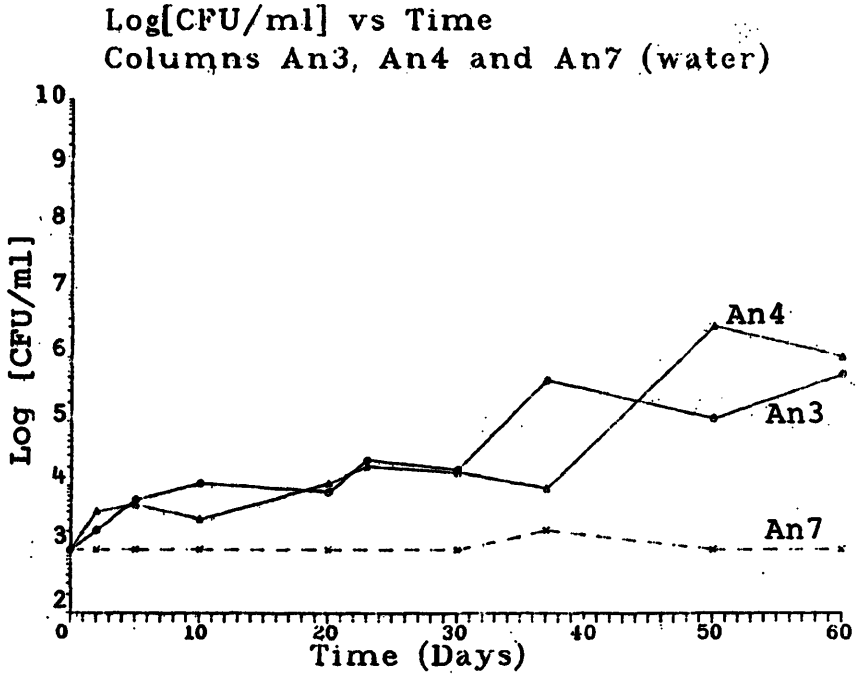
Figure 17a



Relative growth of bacteria above the water-sediment interface in columns An2 and An6.

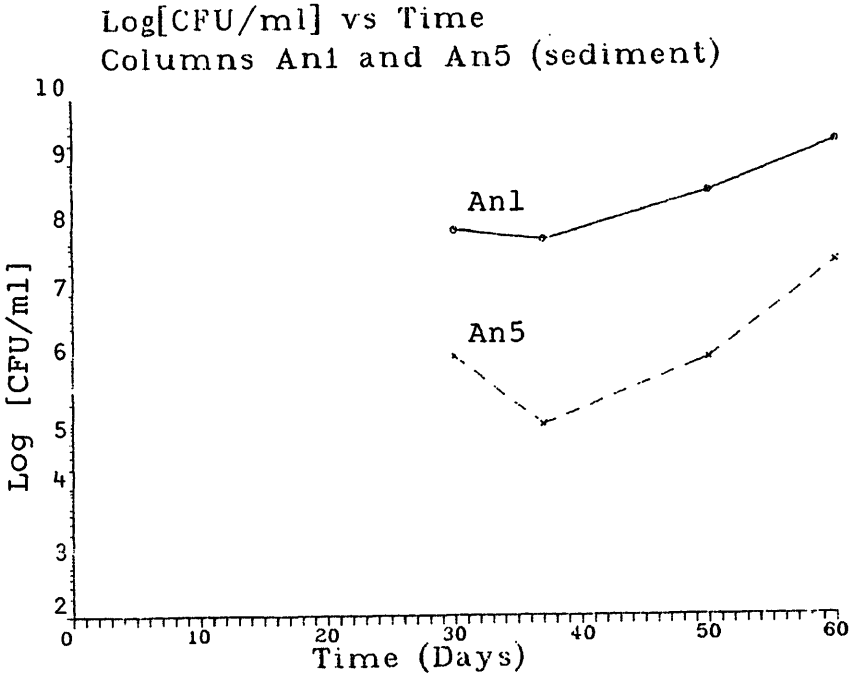
CFU = Colony Forming Units

Figure 17b



Relative growth of bacteria above the water-sediment interface in columns An3 and An4, and An7.
CFU = Colony Forming Units

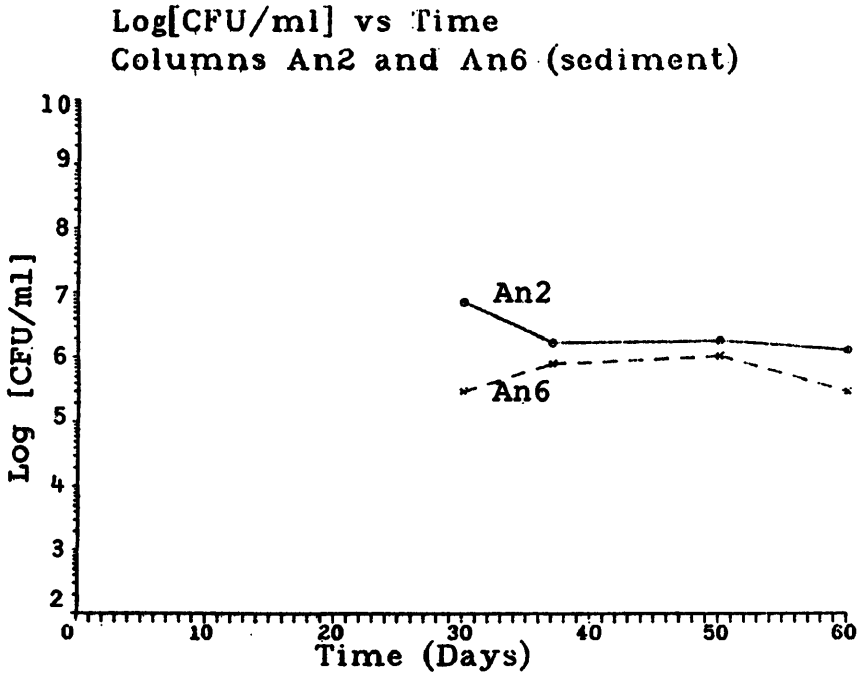
Figure 17c



Relative growth of bacteria below the water-sediment interface in columns AN1 and An5.

CFU = Colony Forming Units

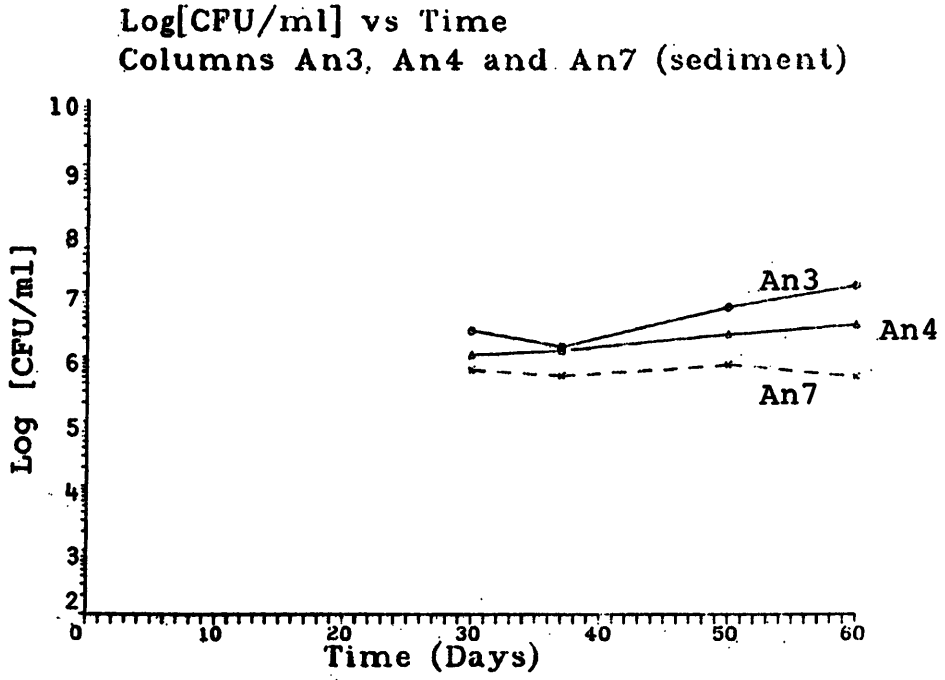
Figure 18a



Relative growth of the bacteria below the water-sediment interface in columns An2 and An6.

CFU - Colony Forming Units

Figure 18b



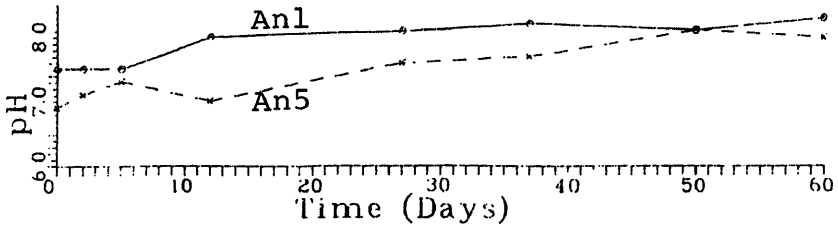
Relative growth of bacteria below the water-sediment interface in columns An3, An4, and An7.

CFU = Colony Forming Units

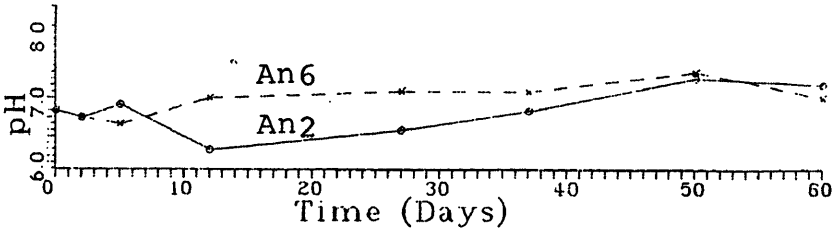
Figure 18c

pH vs Time

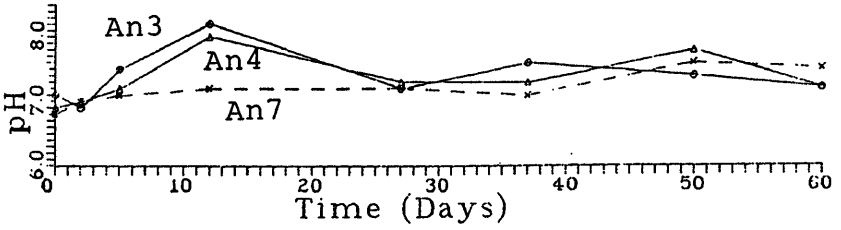
a. Columns An1 and An5



b. Columns An2 and An6

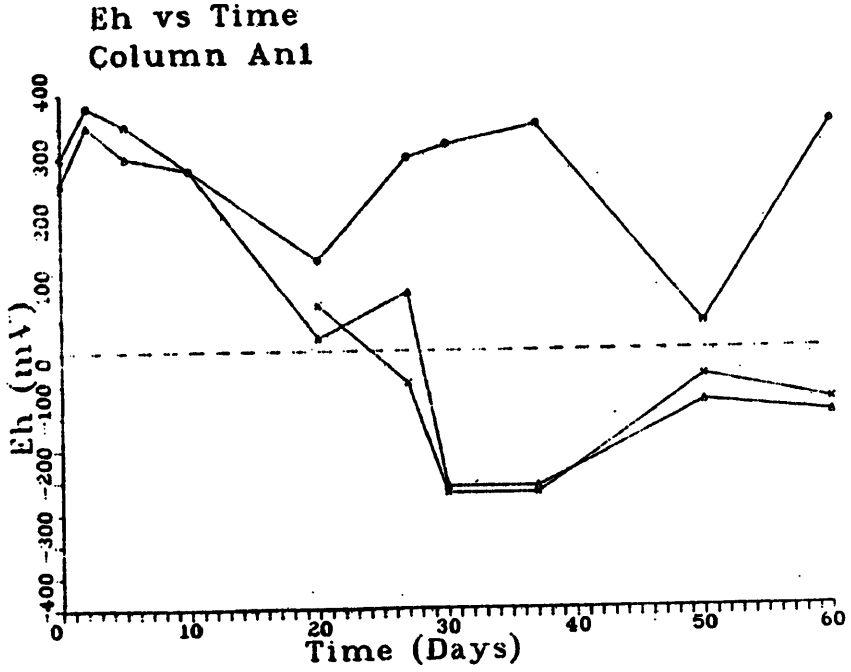


c. Columns An3, An4 and An7



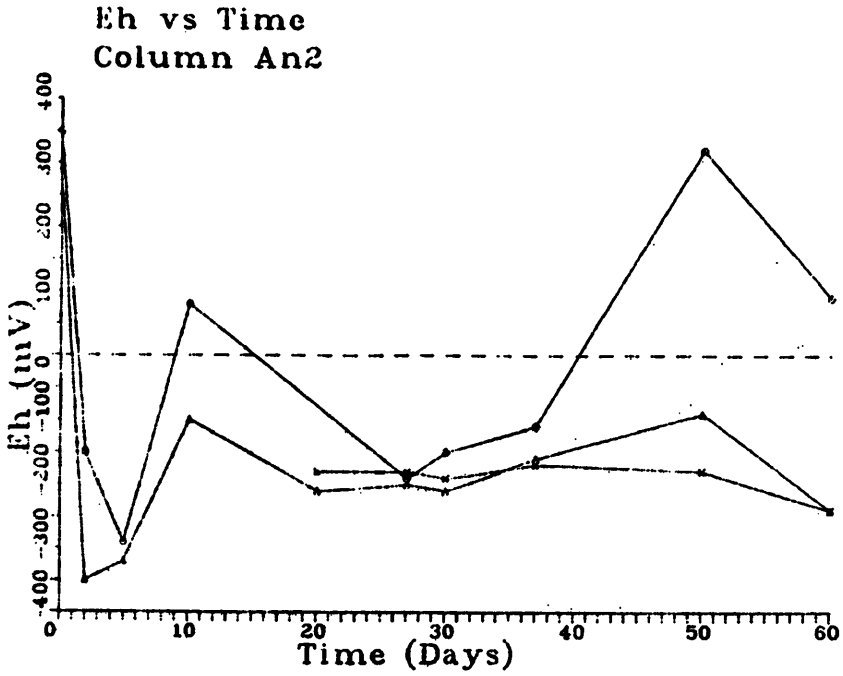
pH of the water in the anaerobic columns vs time.

Figure 19



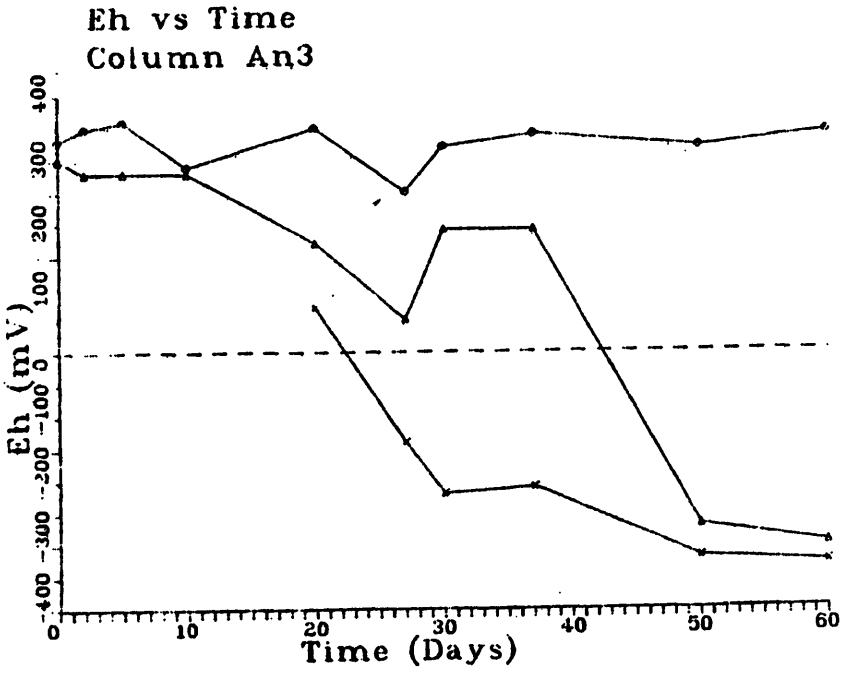
E_h measured at three levels in column An1
o - Water just above the water-sediment interface
Δ - 3-4 cm below the water-sediment interface
x - at the bottom of the column

Figure 20a



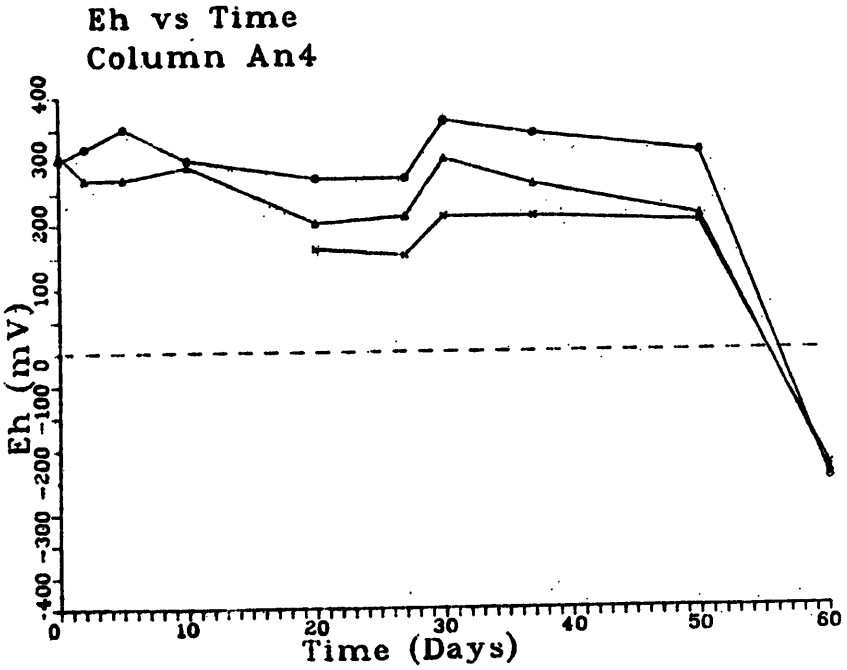
Eh measured at three levels in column An2
o - water just above the water-sediment interface
Δ - 3-4 cm below the water-sediment interface
x - at the bottom of the column

Figure 20b



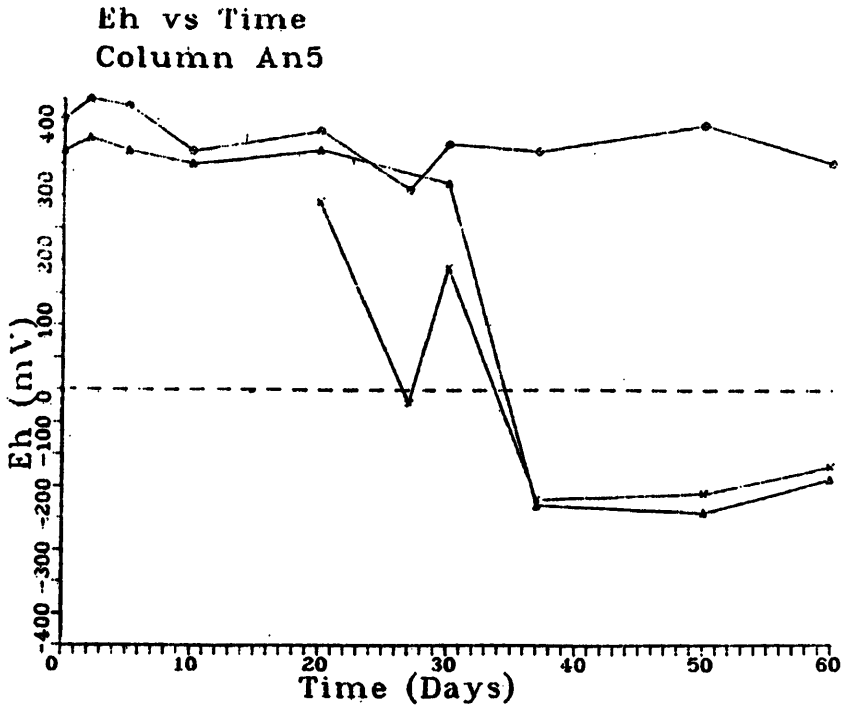
E_h measured at three levels in column An3
o - water just above the water-sediment interface
Δ - 3-4 cm below the water-sediment interface
x - at the bottom of the column

Figure 20c



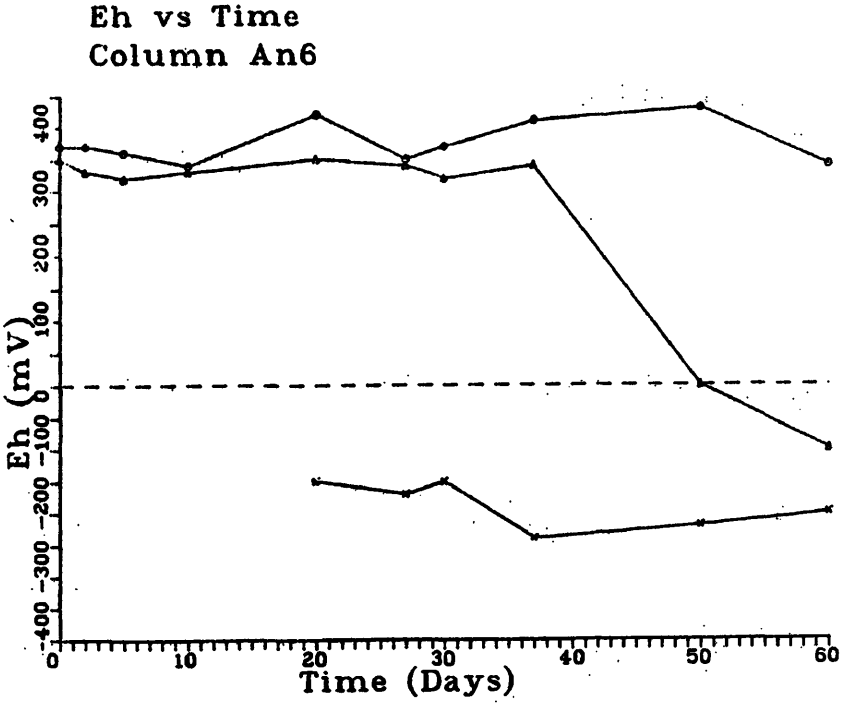
E_h measured at three levels in column An4
o - water just above the water-sediment interface
Δ - 3-4 cm below the water-sediment interface
x - at the bottom of the column

Figure 20d



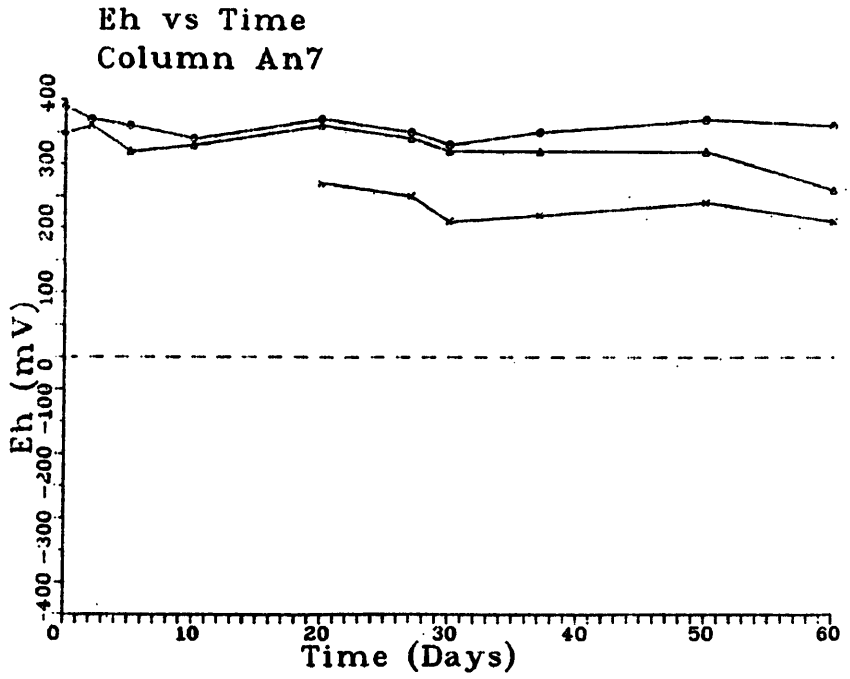
Eh measured at three levels in column An5
o - water just above the water-sediment interface
Δ - 3-4 cm below the water-sediment interface
x - at the bottom of the column

Figure 20e



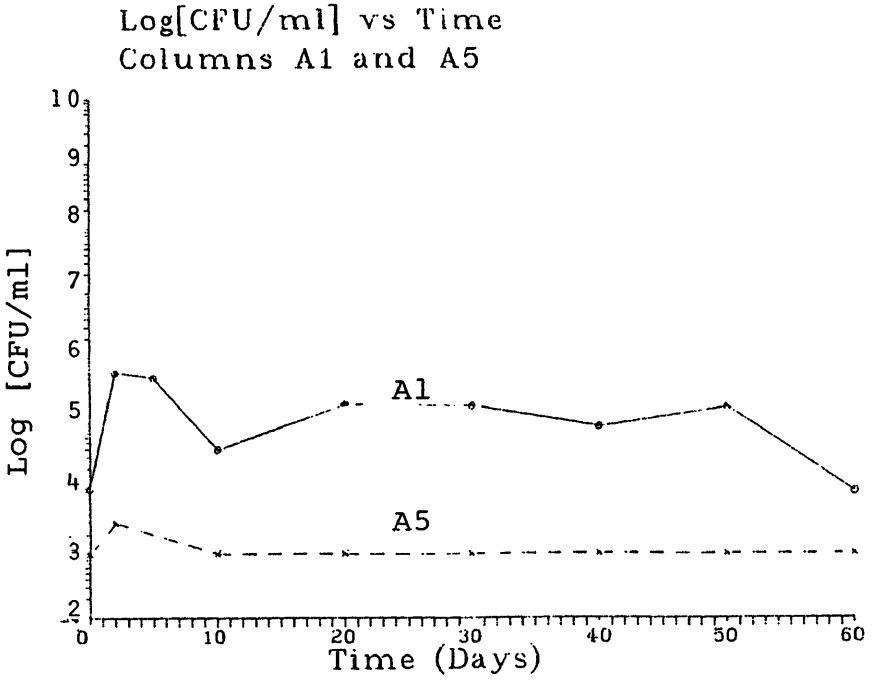
E_h measured at three levels in column An6
o - water just above the water-sediment interface
Δ - 3-4 cm below the water-sediment interface
x - at the bottom of the column

Figure 20f



E_h measured at three levels in column An7
o - water just above the water-sediment interface
Δ - 3-4 cm below the water-sediment interface
x - at the bottom of the column

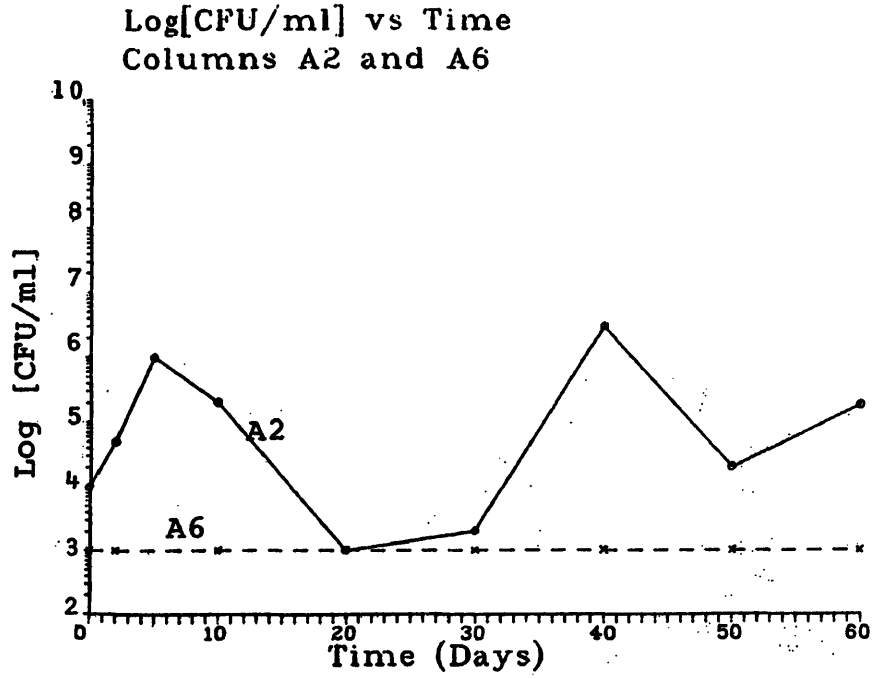
Figure 20g



Relative growth of bacteria in columns A1 and A5. Samples were taken from just above the water-sediment interface.

CFU = Colony Forming Units

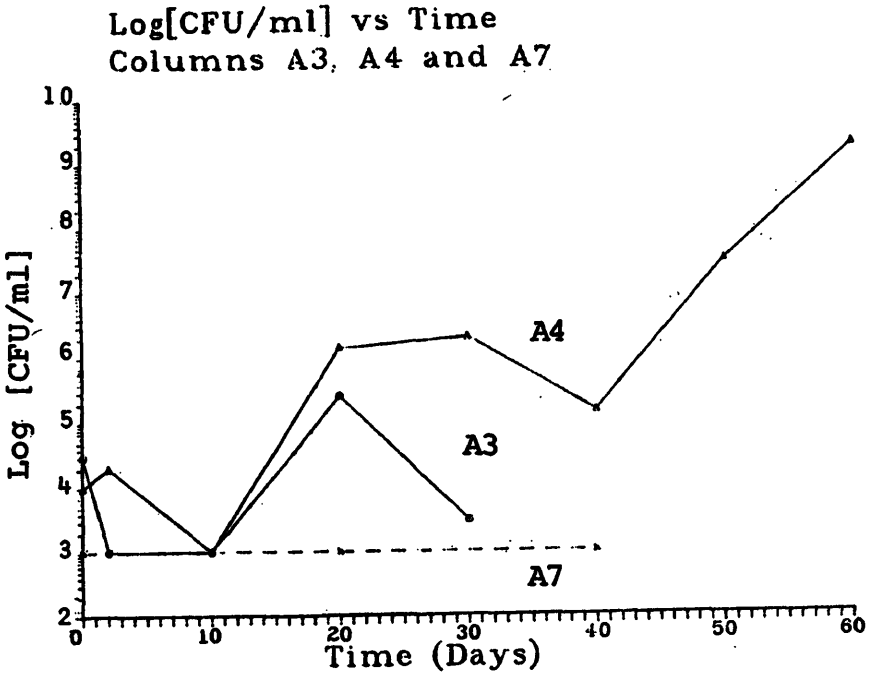
Figure 21a



Relative growth of bacteria in columns A2 and A6. Samples were taken from just above the water-sediment interface.

CFU = Colony Forming Units

Figure 21b



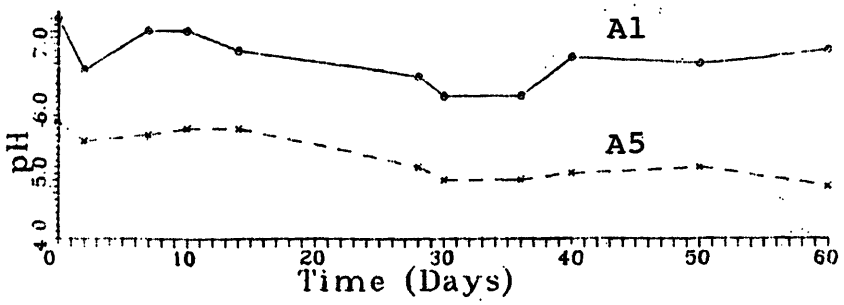
Relative growth of bacteria in columns A3, A4, and A7. Samples were taken from just above the water-sediment interface.

CFU = Colony Forming Units

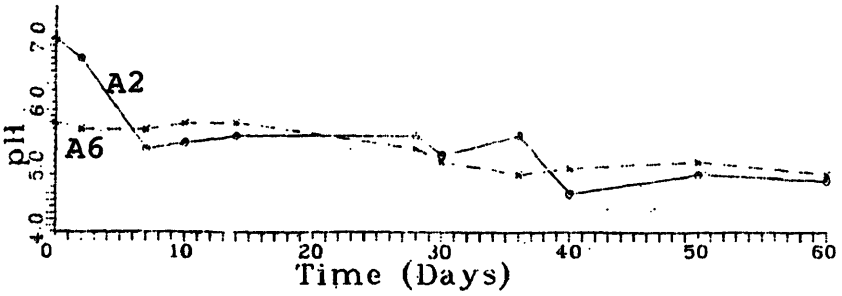
Figure 21c

pH vs Time

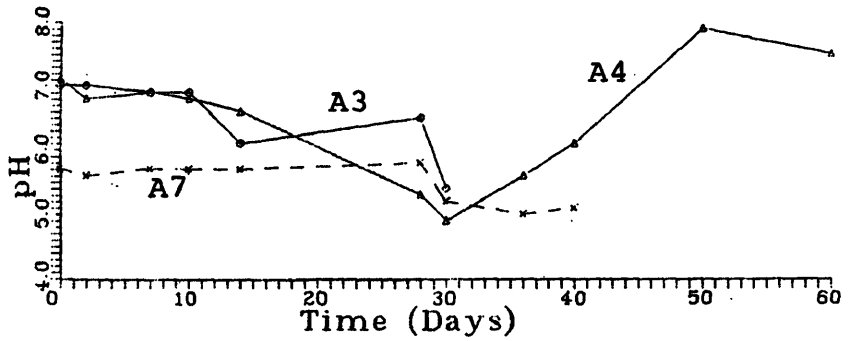
a. Columns A1 and A5



b. Columns A2 and A6



c. Columns A3, A4 and A7



Plots of pH against time of incubation. The pH was measured just above the water-sediment interface.
a. Columns A1 and A5; b. Columns A2 and A6; c. Columns A3, A4, and A7.

Figure 22

Gas Chromatography-Mass Spectrometry of the Sediment Extracts:

General Comments

The heptane, benzene, chloroform, and methyl ester fractions from the separations performed on the sediment extracts of columns An1, An2, An3, An4, and An7; and A1, A2, A3, A4, and A7 were analyzed by gas chromatography-mass spectrometry (GC-MS) The results are catalogued below.

Heptane and Benzene Fractions

Figure 23 (a and b) shows the Total Ion Current (TIC) and the 189 and 191 mass chromatograms for the heptane fraction of column An1. The mass spectra of peaks H3, H4, and the sixth component of the starting material are shown in Figures 27 and 28. The TIC 189 and 191 mass chromatograms of the heptane fraction of column An7 are given in Figure 29. The five peaks of interest, H5-H9, have their mass spectra shown in Figures 30-33. Peak H7 has its mass spectrum compared with that of H1 (column An1) in Figure 31, and the mass spectrum of peak H9 is shown with that of the sixth component of the starting material in Figure 33. Figure 34 gives the TIC and 191 mass chromatogram of column A4. The mass spectrum of peak H1a is shown in Figure 35 as is the mass spectrum of the sixth component of the starting material.

Figure 36 is the TIC and 205 mass chromatogram of the benzene fraction of column An7. The mass spectrum of the major 205 ion of Figure 36, peak B1, is shown in Figure 37a. Figure 37b is the mass spectrum of the sixth component of the starting material.

The TIC and 205 mass chromatogram of the benzene fraction of column A3 are shown in Figure 38. The mass spectra of peak B1a and the first component of the starting material are given in Figure 39. Figure 40 shows the TIC and 205 mass chromatogram of the benzene fraction of column An4. Peak B2a has its mass spectrum compared to that of the fifth component of the starting material in Figure 41.

Chloroform Fractions

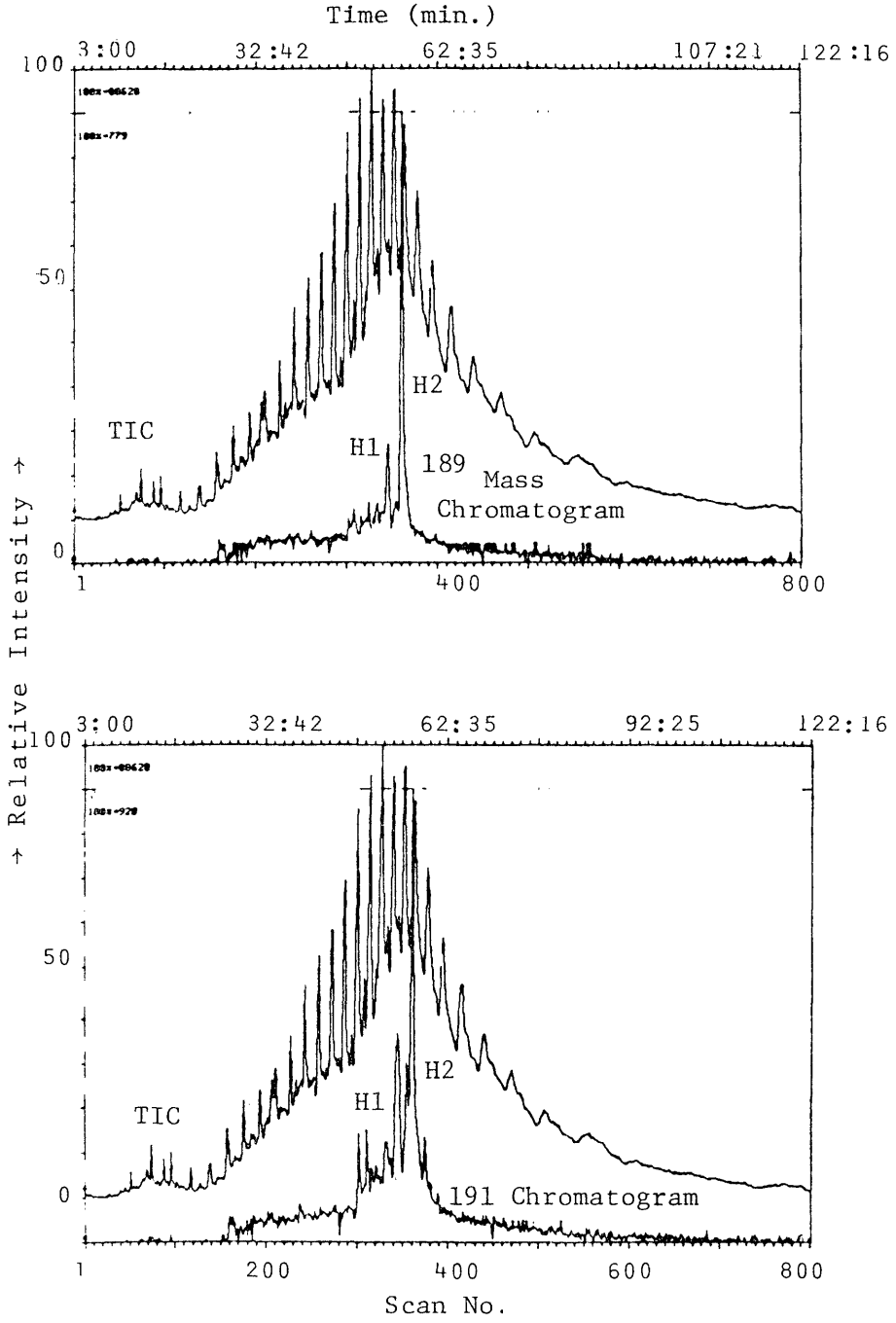
Figure 42a shows the TIC and 205 mass chromatogram of the chloroform fraction of column An4. Figure 42b is the starting material analyzed on the same GC column using a different temperature program (see caption) The mass spectra of peaks C1-C3 are shown in Figures 43-45, along with the spectra of the starting materials they represent. Peak C4 has its mass spectrum given in Figure 46a. A generalized structure of the compound represented by peak C4 is given in Figure 46b.

Methyl Ester Fractions

The TIC and 235 mass chromatogram of the methyl esters

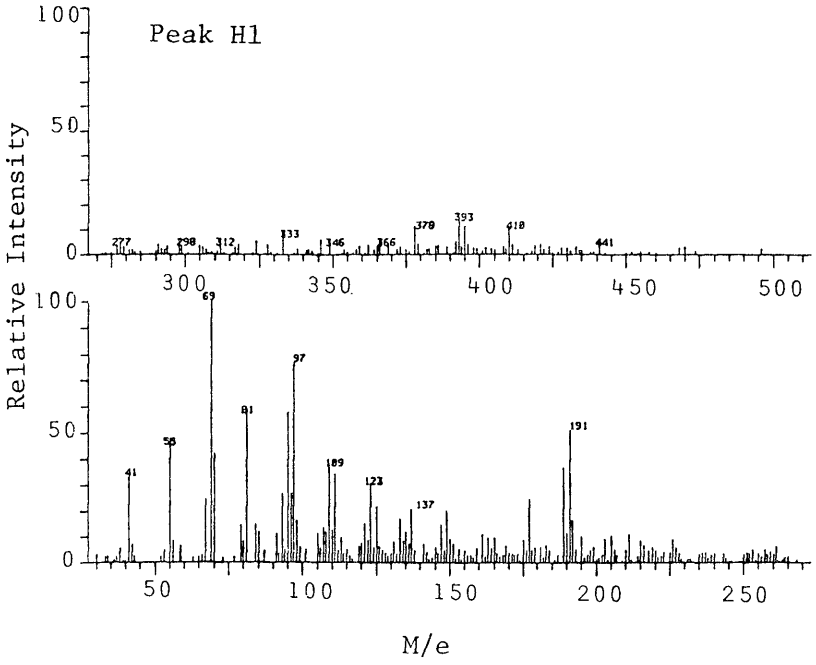
of column A3 are shown in Figure 47a. The mass spectrum of peak Mla is given in Figure 47b. Figure 48 shows the TIC and 191 and 205 mass chromatograms of the methyl ester fractions of column An3. Figure 49 gives the mass spectrum of peak Ml, together with that of peak 1 of the starting material. The mass spectrum of peak M2 is shown in Figure 50. (The spectra of all starting materials are from Dr. D. Miiller, Phillips Petroleum.)

a.



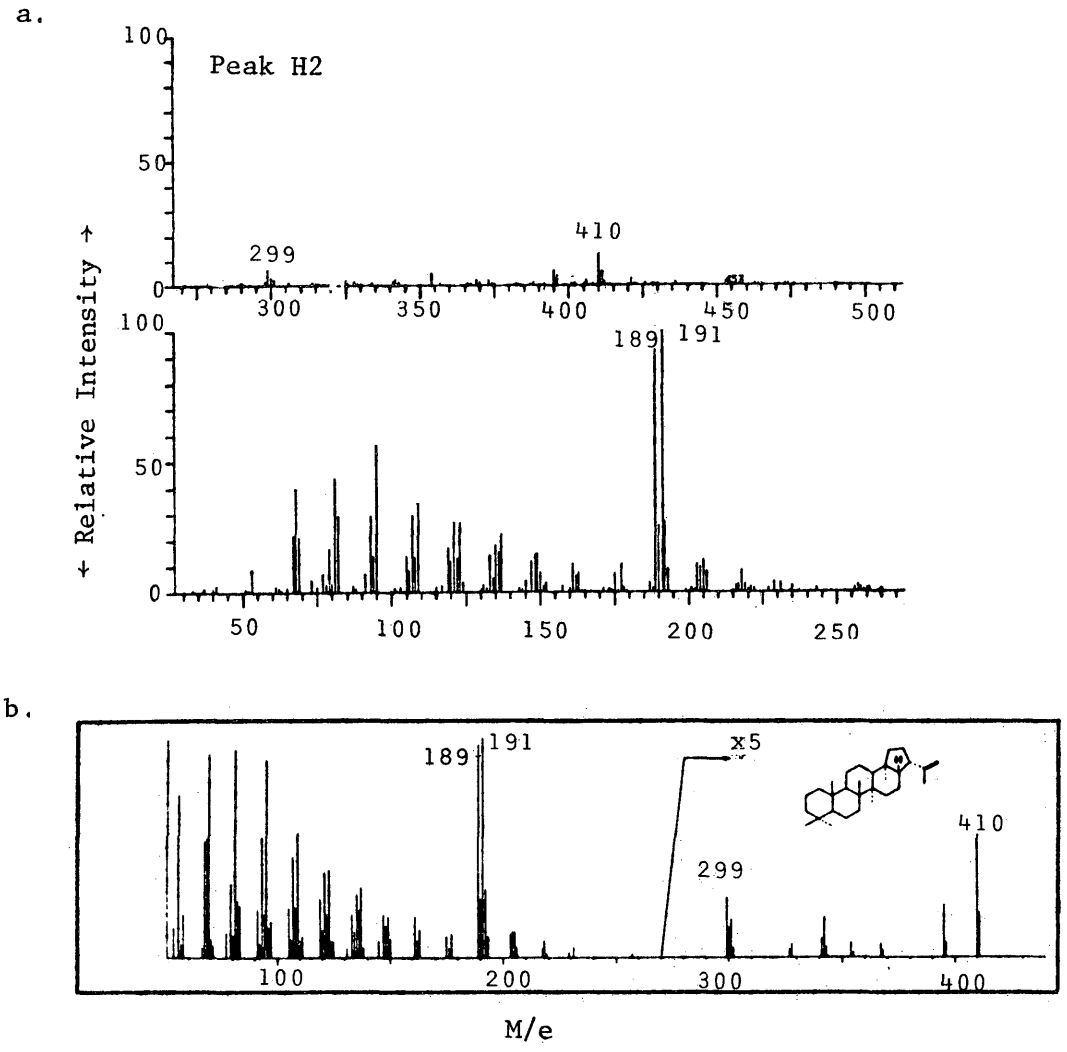
Total Ion Current (TIC) and Mass Chromatograms of the heptane fraction of column An1; (a) TIC and 189 mass chromatogram; (b) TIC and 191 mass chromatogram (GC parameters: 12.5 M x 0.25 mm SE-52 WCOT; 50-280°C at 4°C/min; 1.0 ml He/min.).

Figure 23



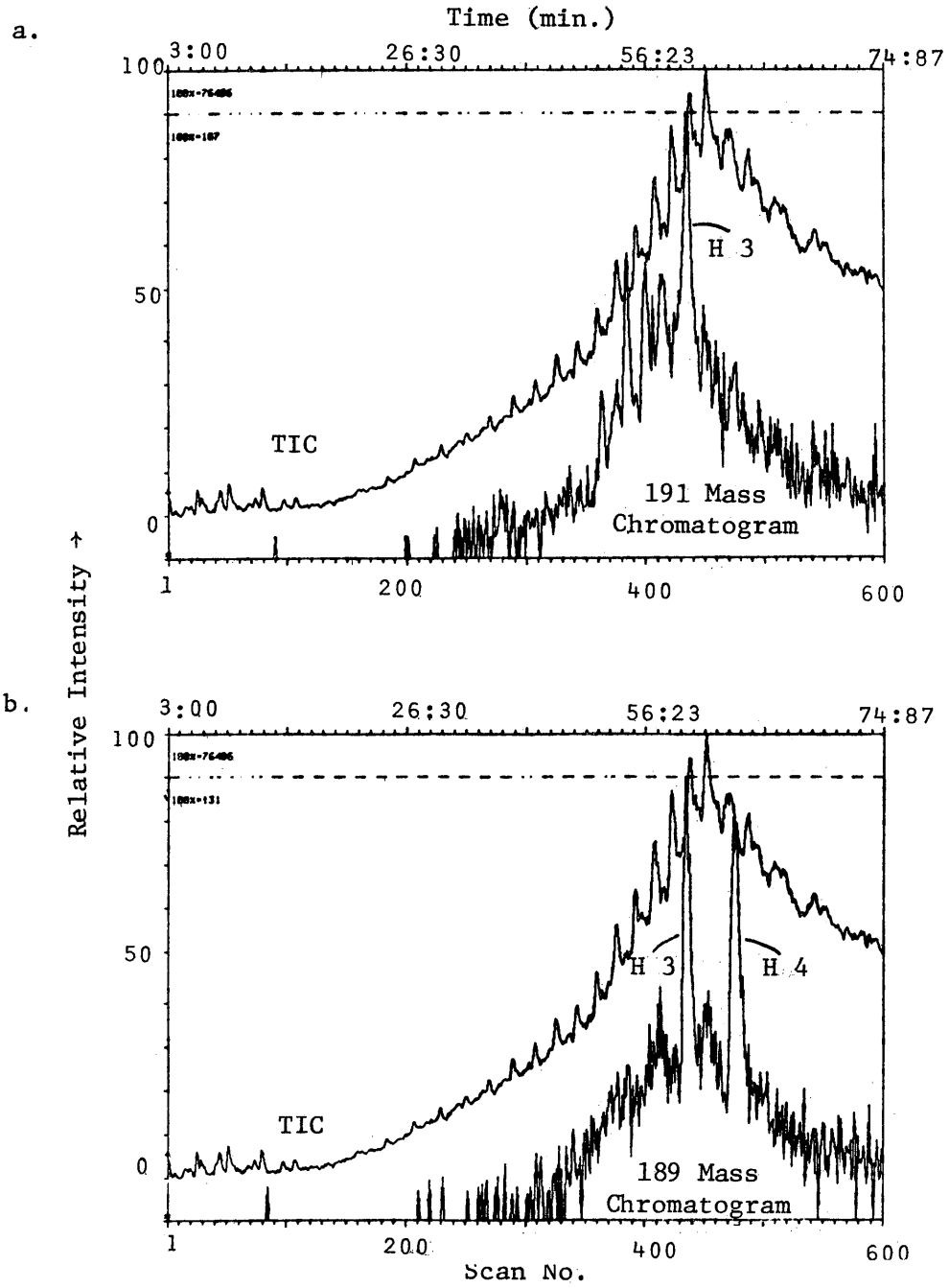
Mass Spectrum of peak H1 (Column An1, heptane fraction).

Figure 24



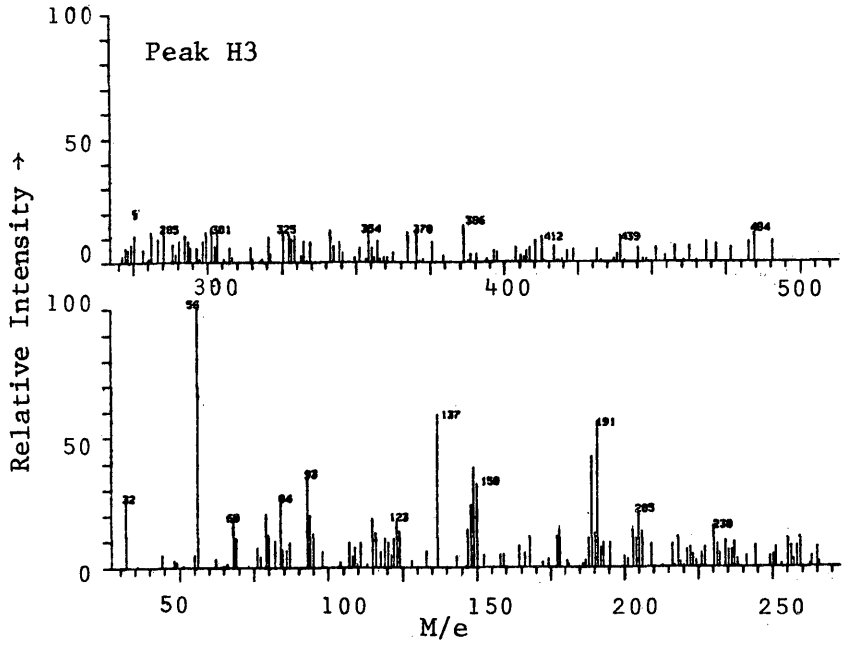
Mass spectrum of peak H2(a) (column An1, heptane fraction) compared with that of diploptene (b) (Dastillung, 1976, p. 60). Diploptene is indigenous to the anoxic marine sediment.

Figure 25



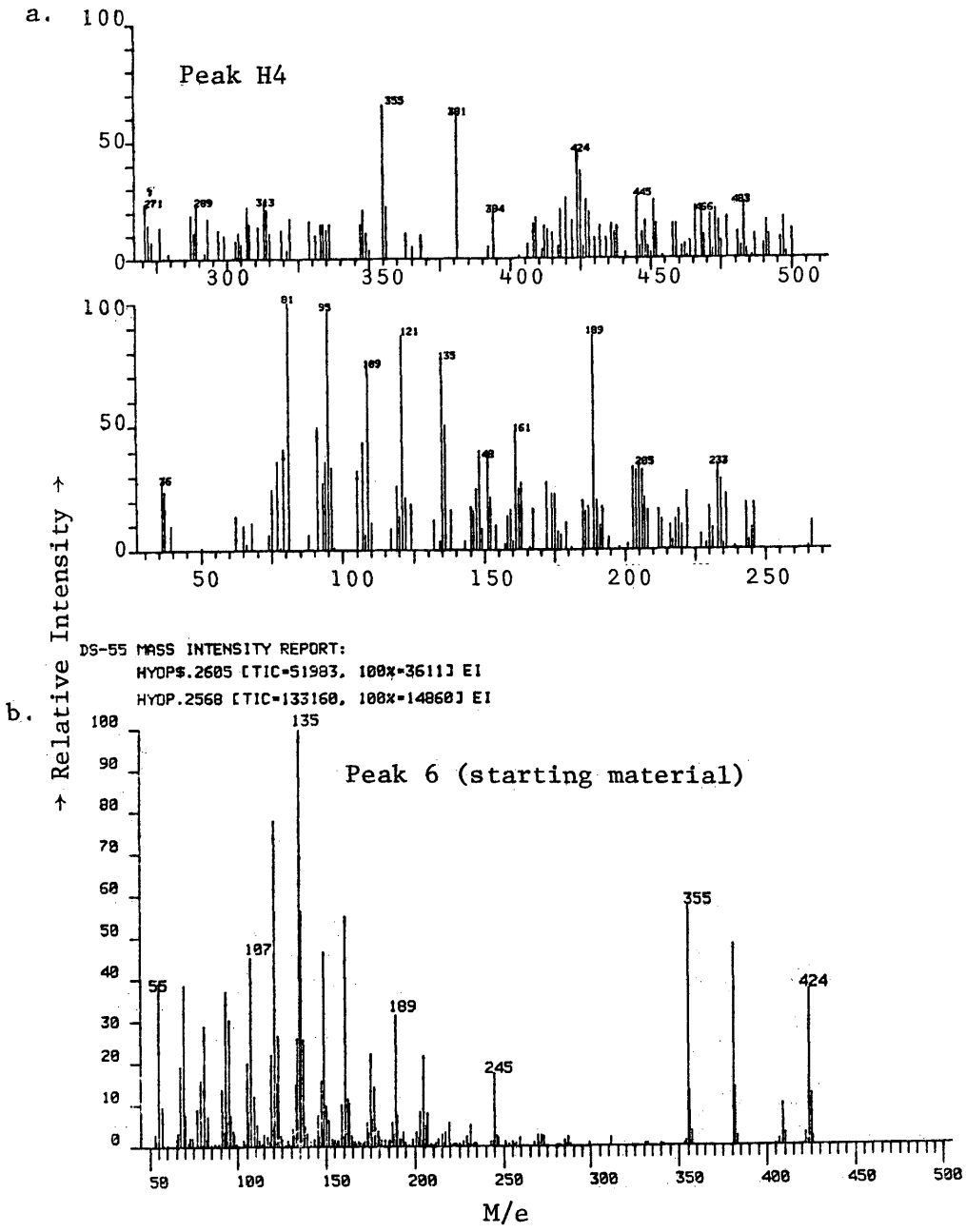
Total Ion Current (TIC) and mass chromatograms of the heptane fraction of column An4; (a) TIC and 191 mass chromatogram; (b) TIC and 189 mass chromatogram (GC parameters: 12.5 m x 0.25 m SE-52 WCOT; 60-280°C at 4°C/min.; 1.0 ml He/min.).

Figure 26



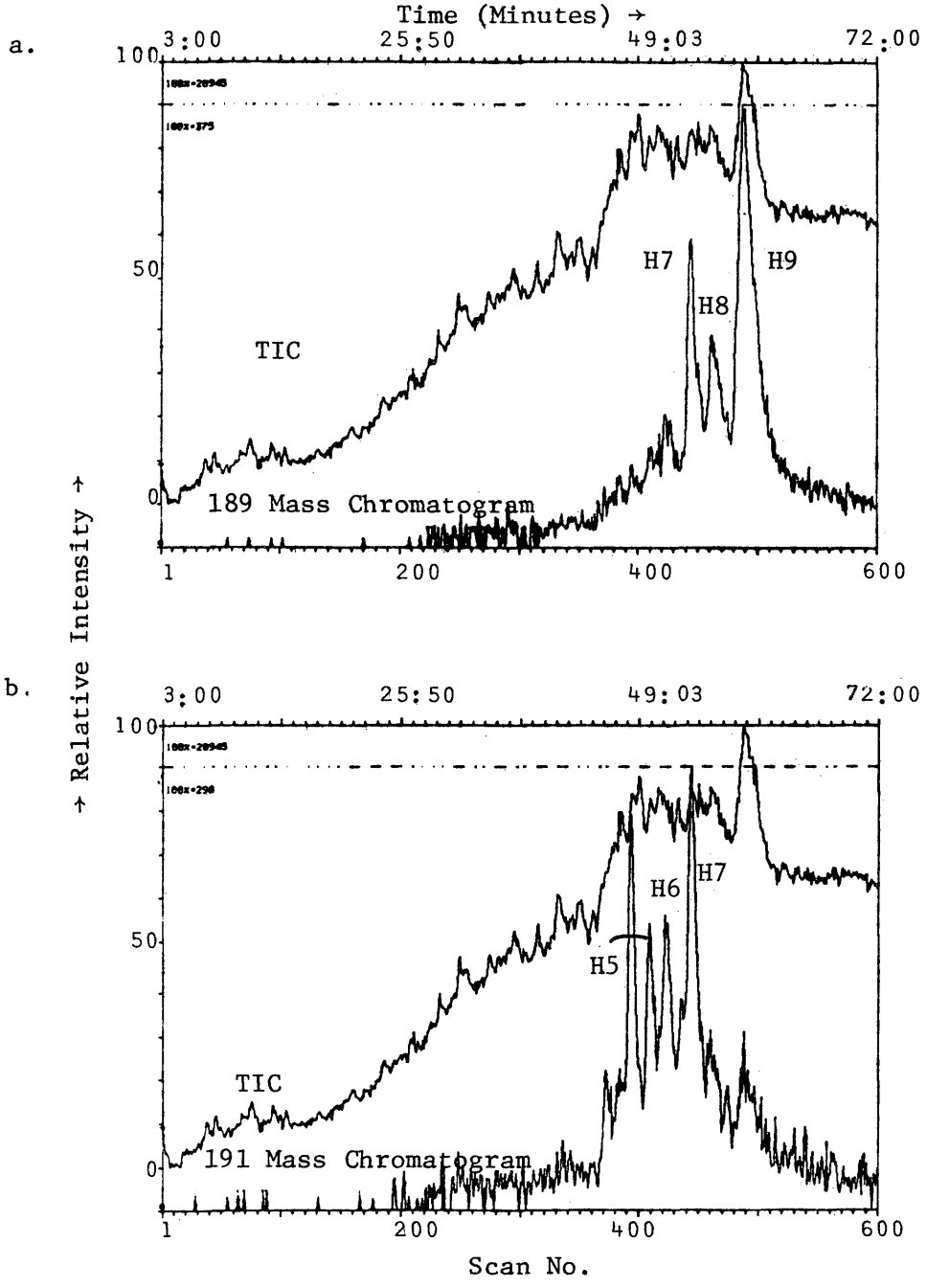
Mass spectrum of peak H3 (column An4, heptane fraction).
(Mass spectrum is poor due to low abundance of this material.)

Figure 27

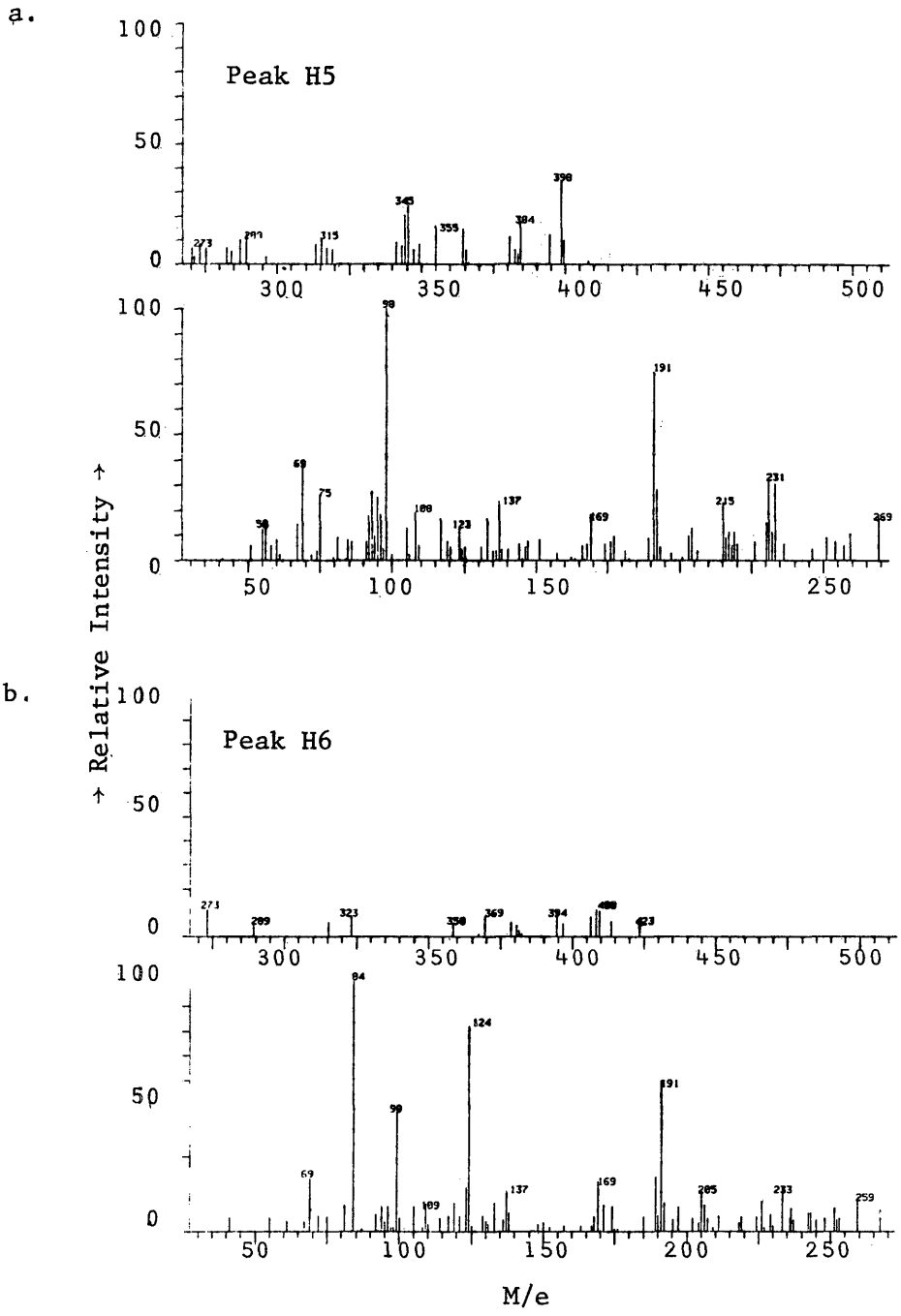


Mass spectrum of peak H4 (a) (column An4, heptane fraction) compared with that of the sixth component of the starting material (b). (Mass spectrum of H4 is poor because of coelution and low abundance of this material.)

Figure 28

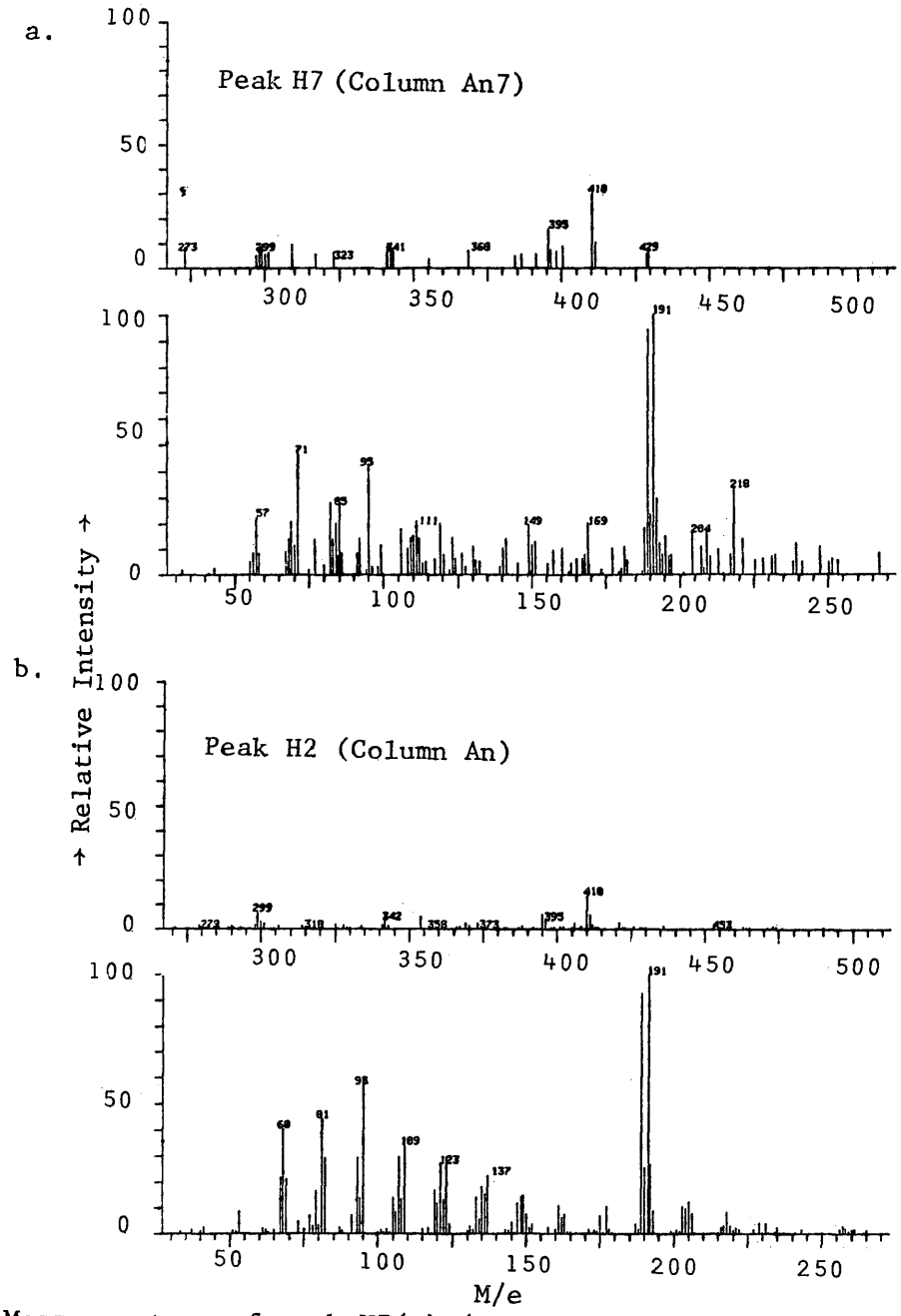


TIC and mass chromatograms of heptane fraction of column An7; (a) TIC and 189 mass chromatogram; (b) TIC and 191 mass chromatogram. (GC parameters 12.5 M x 0.25 mm SE-52 WCOT; 60-280°C at 4°C/minute; 1.0 ml He/minute). Figure 29



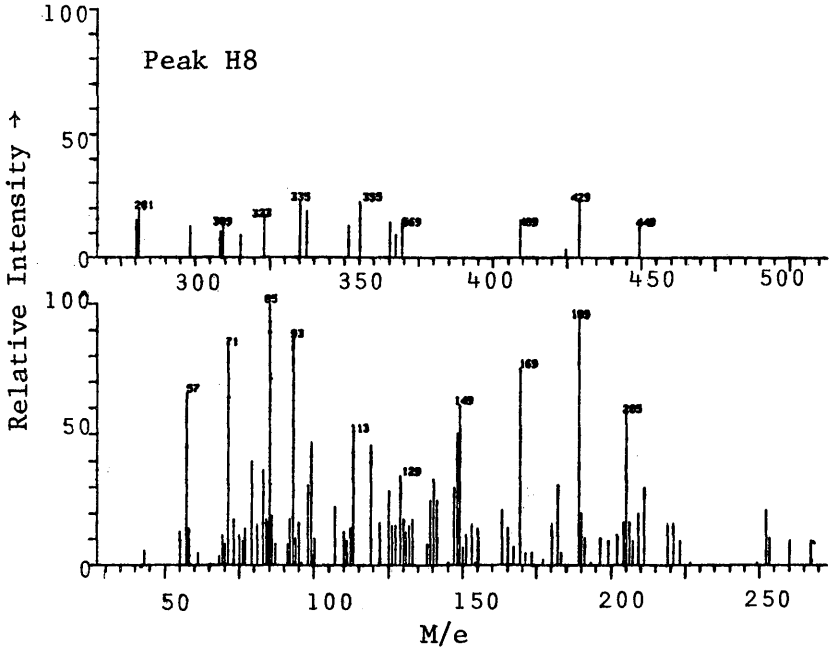
Mass spectra of peak H5 (a) and peak H6 (b) (Column An7, heptane fraction).

Figure 30



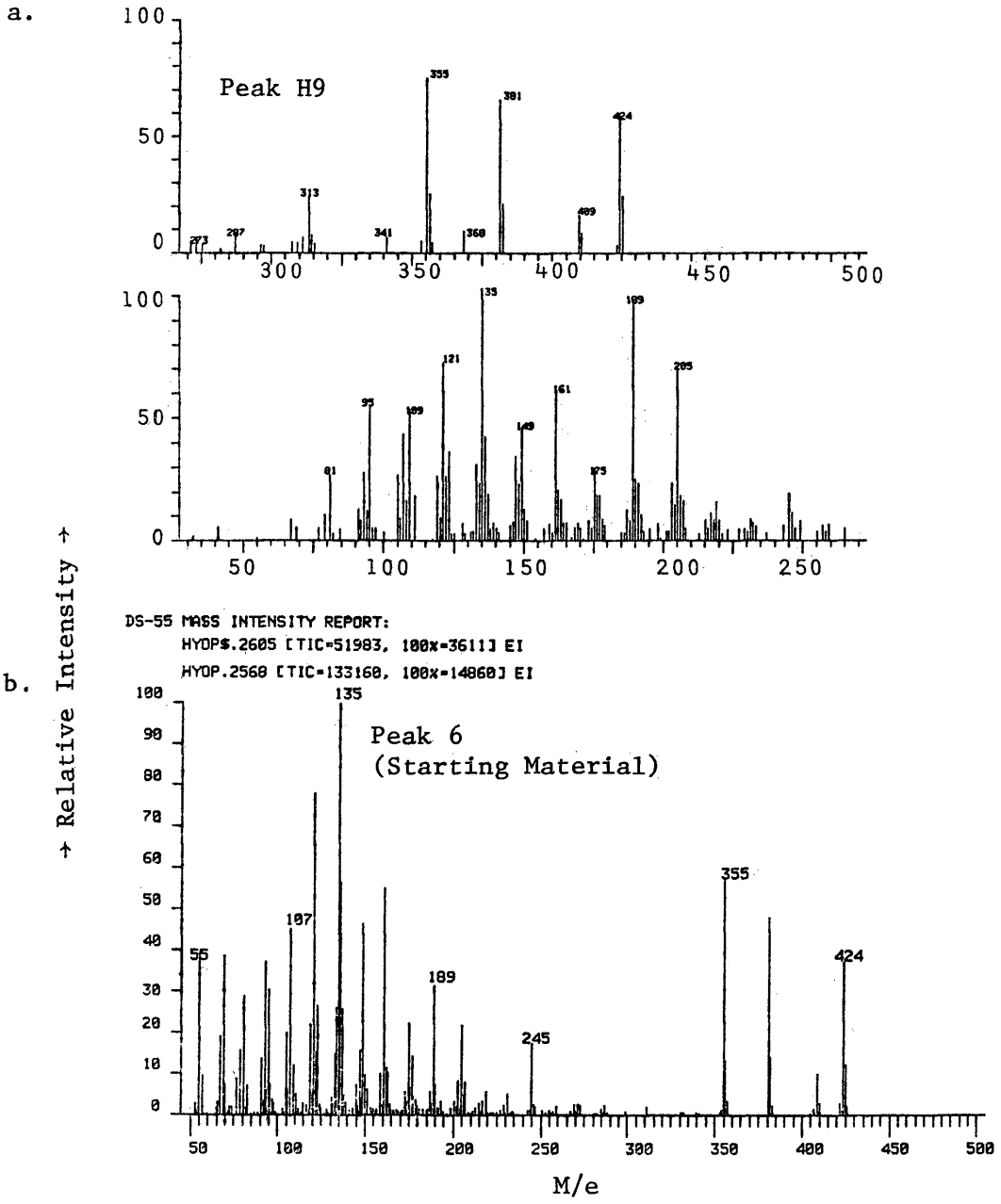
Mass spectrum of peak H7(a) (Column An7, heptane fraction) compared with that of Peak H2(b) (Column An1, heptane fraction). Both of peaks are thought to represent dipoptene which is indigenous to the sediment.

Figure 31



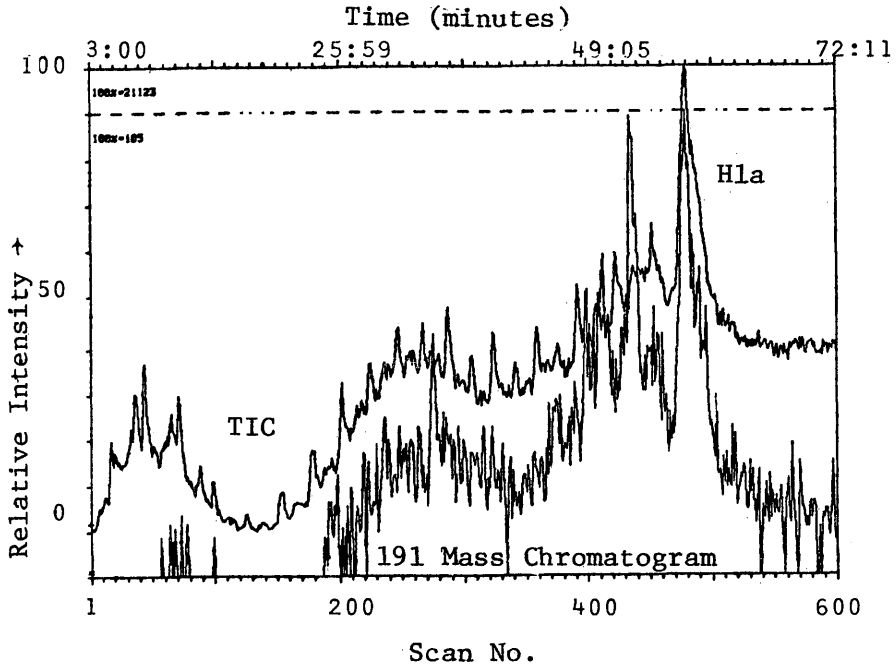
Mass spectrum of Peak H8 (Column An7, heptane fraction).

Figure 32



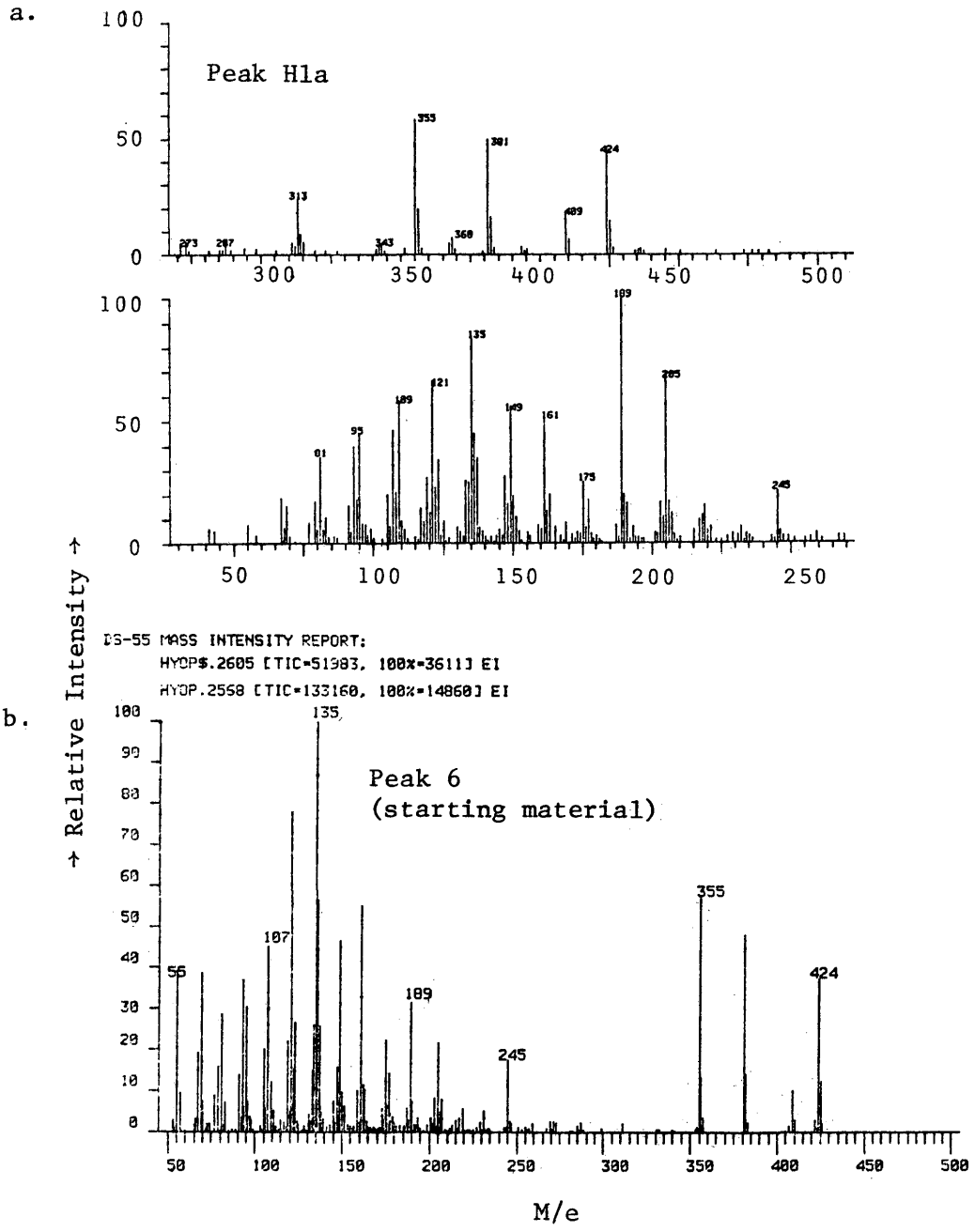
Mass spectrum of Peak H9(a) (Column An7, heptane fraction) compared with that of the sixth component of the starting material (b).

Figure 33



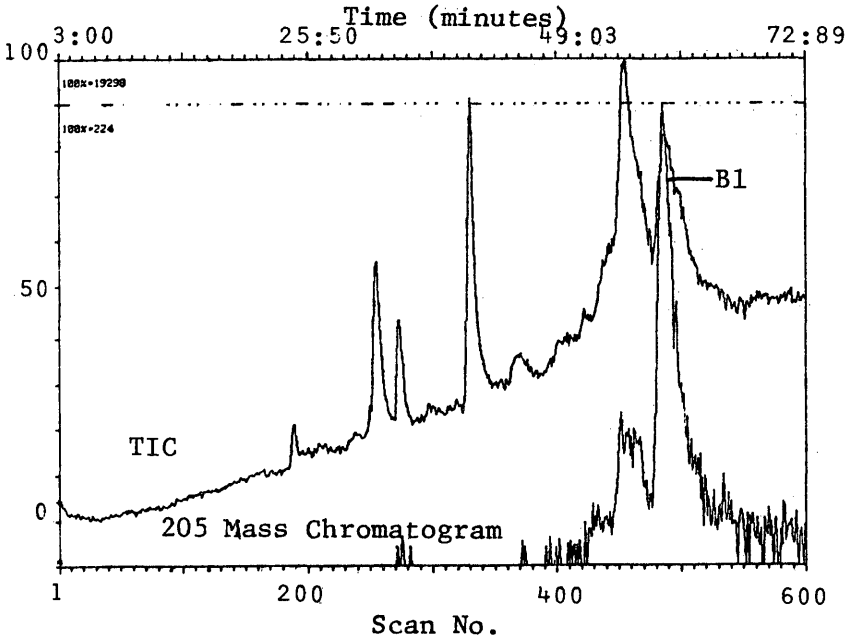
Total Ion Current (TIC) and 191 mass chromatogram of the heptane fraction of Column A4. (GC parameters 12.5 M x 0.25 mm SE-52 WCOT; 60-280°C at 4°C/min.; 1.0 ml He/min.)

Figure 34



Mass spectrum of peak Hla(a) (Column A4, heptane fraction) compared with that of Peak 6 of the starting material (b).

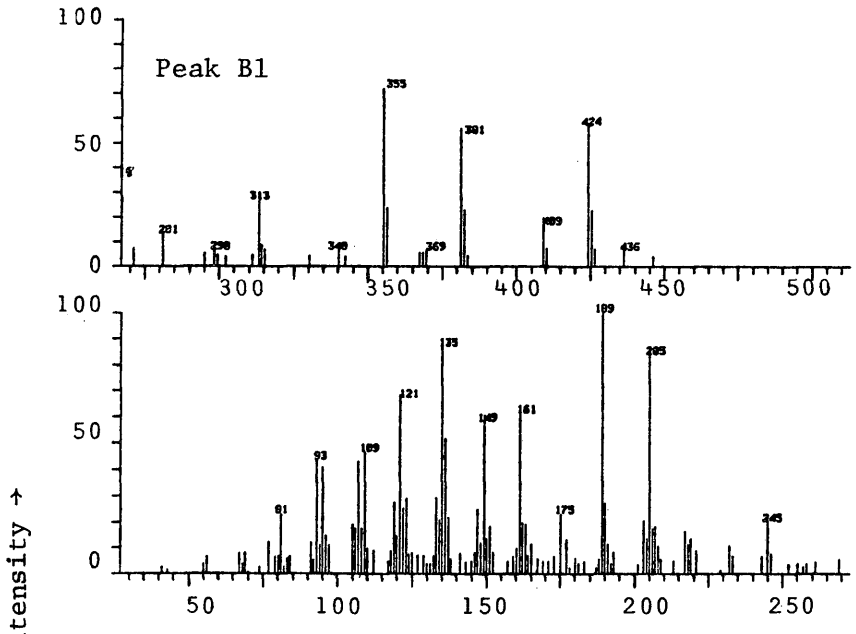
Figure 35



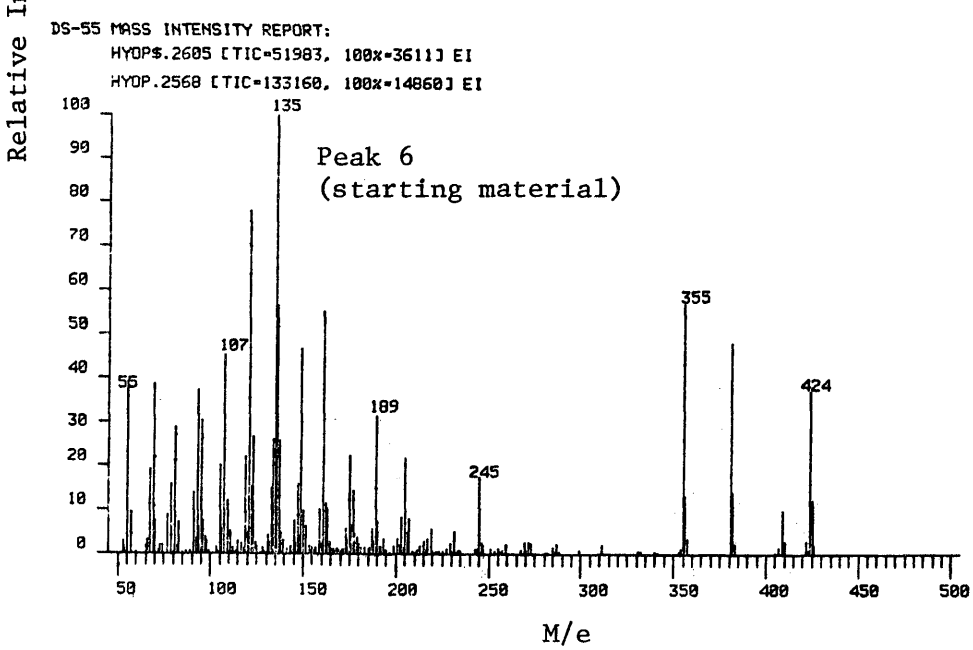
TIC and 205 mass chromatogram of the benzene fraction of Column An7. (GC parameters: 12.5M x 0.25 mm SE-52 WCOT; 60-280°C at 4°C/min.; 1.0 ml He/min.)

Figure 36

a.

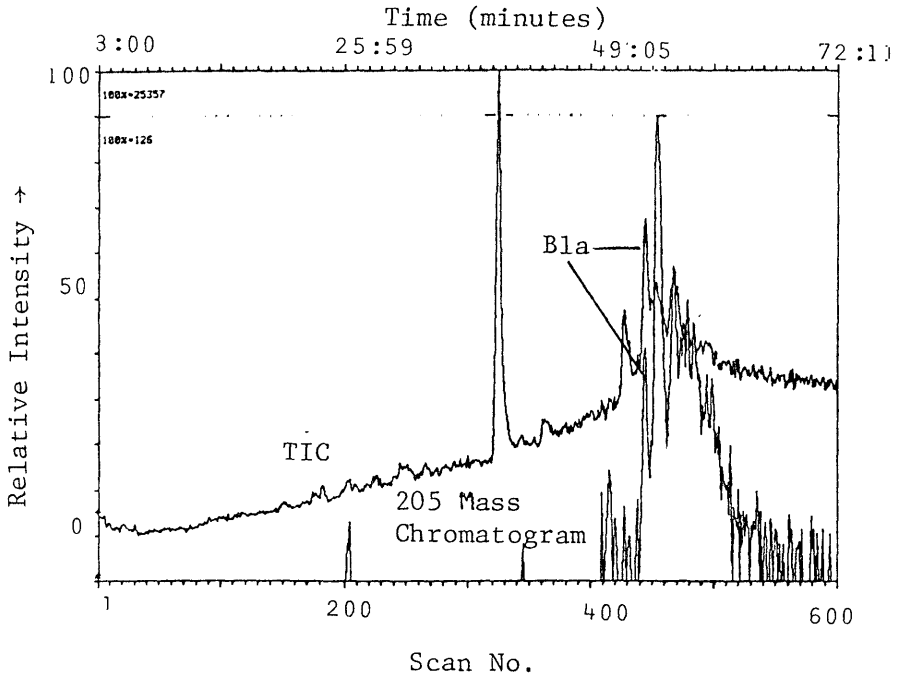


b.



Mass spectrum of Peak B1(a) (Column An7, benzene fraction) compared with that of the sixth component of the starting material (b).

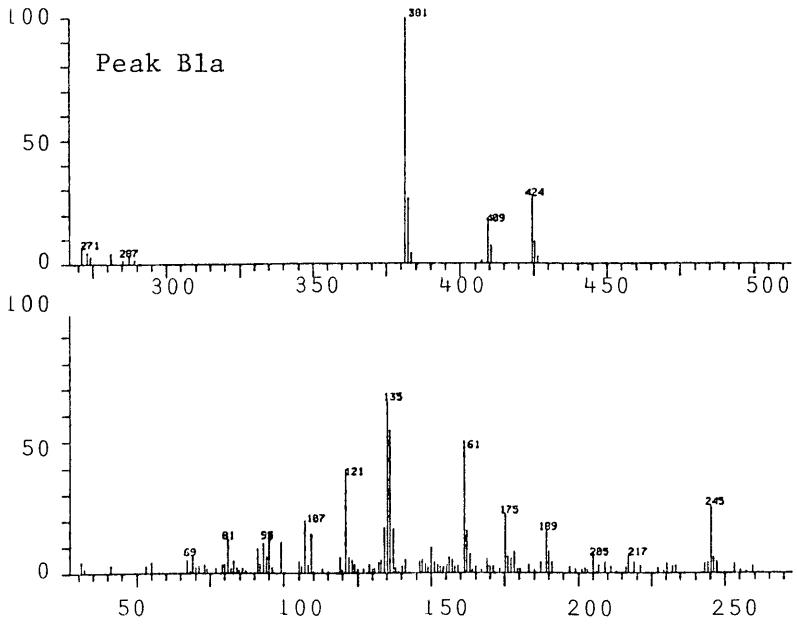
Figure 37



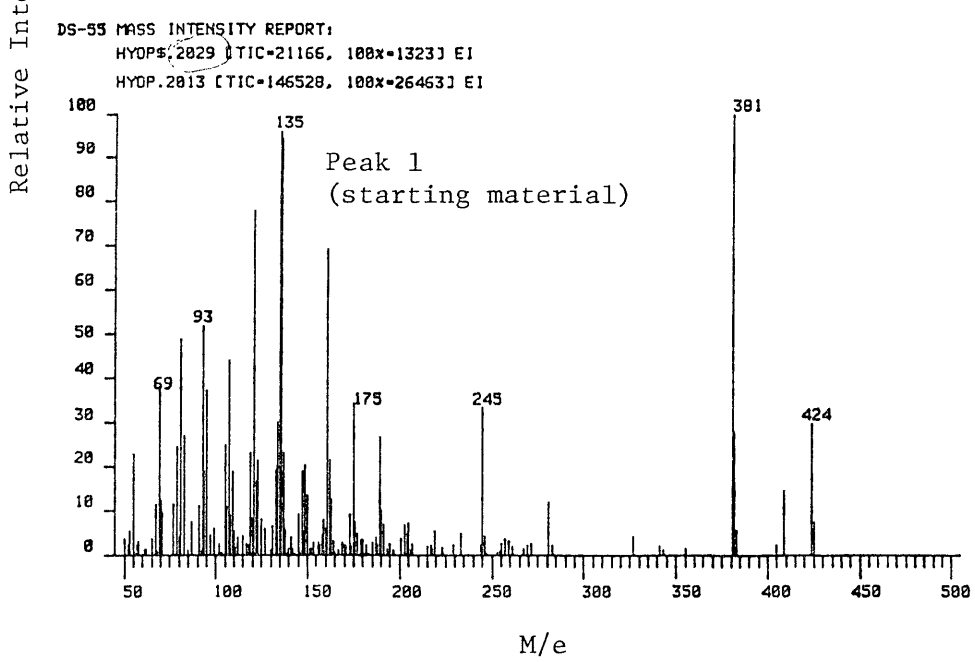
TIC and 205 mass chromatogram of the benzene fraction of Column A3. (GC parameters: 12.5 M x 0.25 mm SE-52 WCOT; 60-280°C at 4°C/min.; 1.0 ml He/min.)

Figure 38

a.

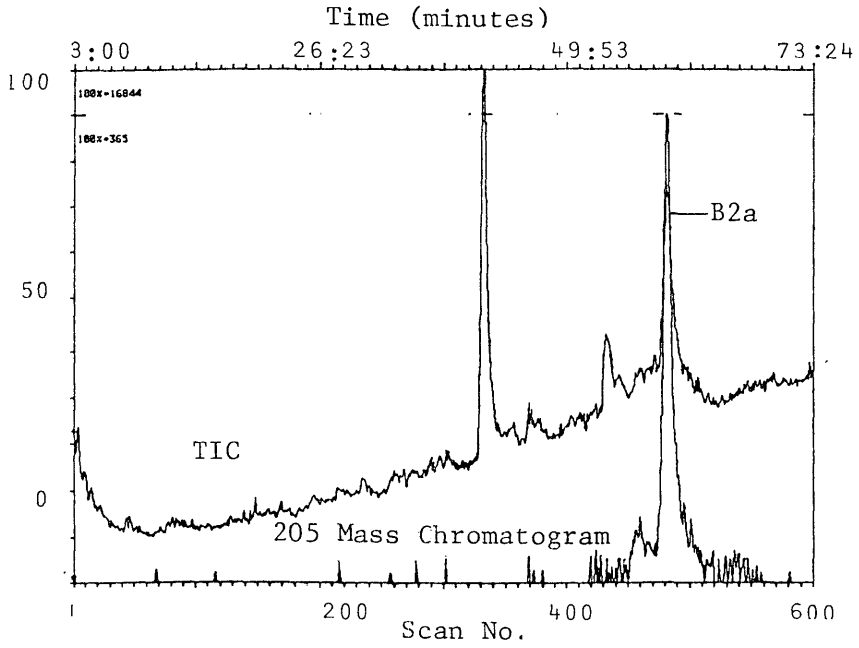


b.



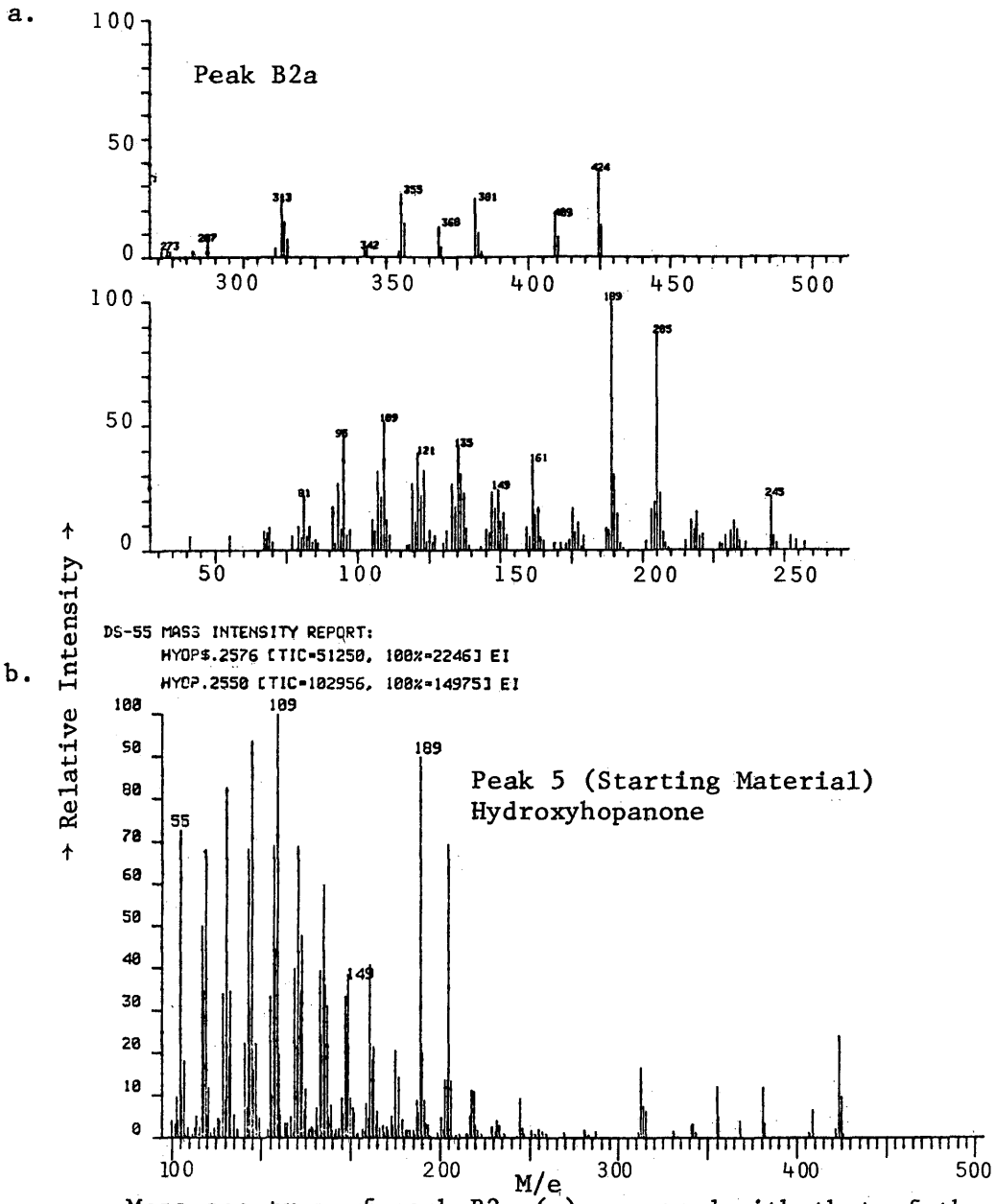
Mass spectrum of peak Bla (a) compared with that of the first component of the starting material (b).

Figure 39



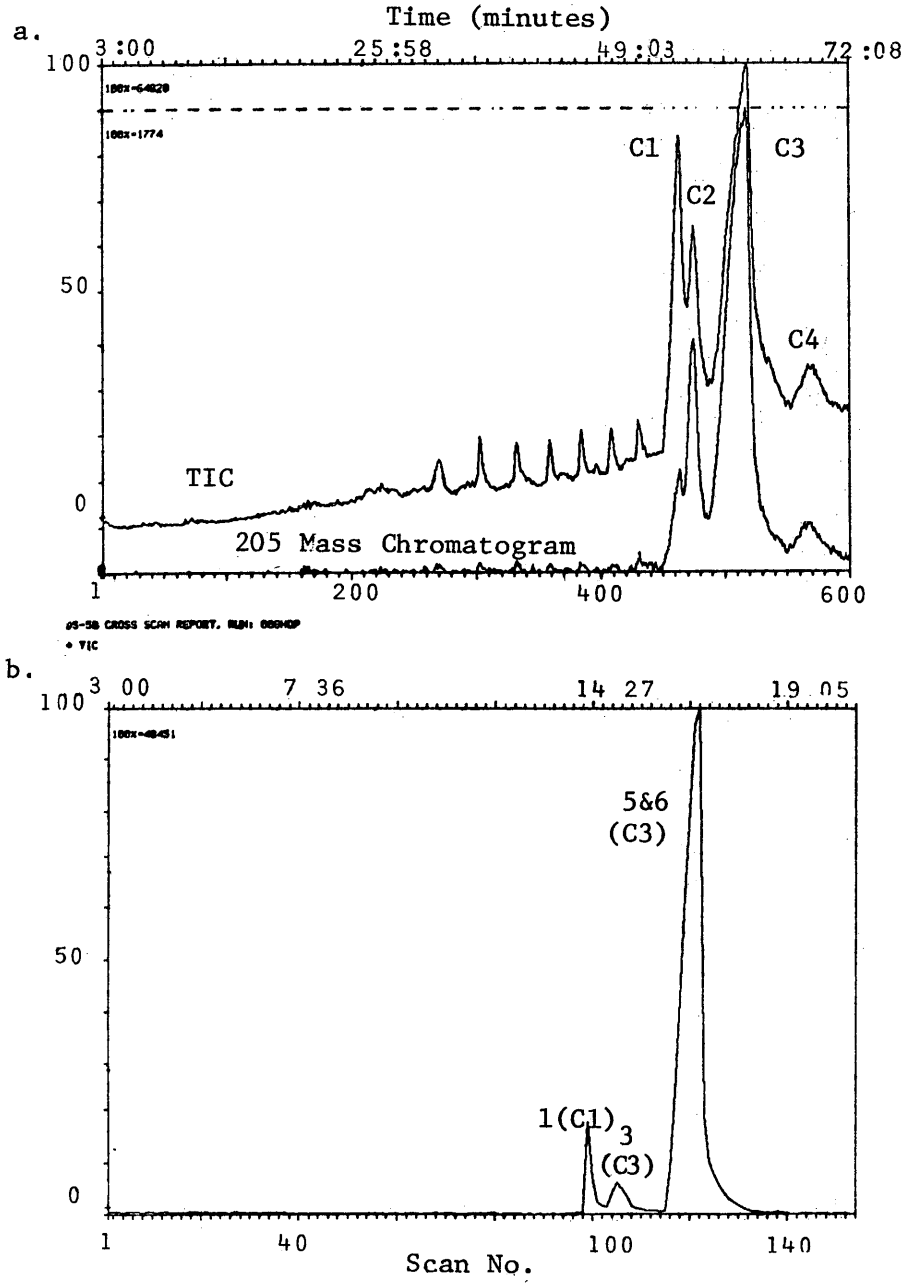
TIC and 205 mass chromatogram of the benzene fraction of Column A4. (GC parameters: 12.5 M x 0.25 mm SE-52 WCOT; 60-280°C at 4°C/min.; 1.0 md He/min.)

Figure 40



Mass spectrum of peak B2a (a) compared with that of the fifth component of the starting material (b).

Figure 41

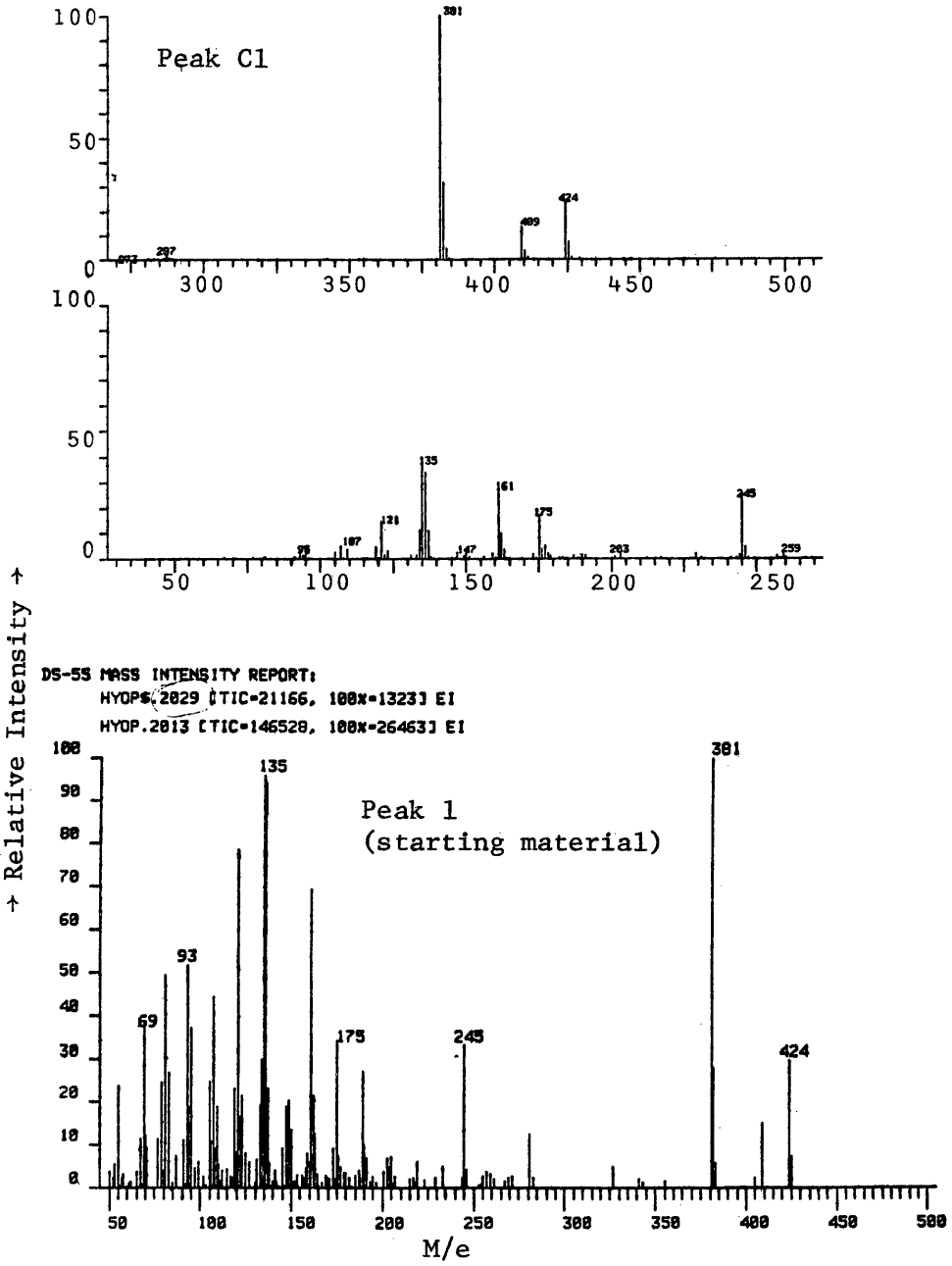


a. TIC and 205 mass chromatogram of chloroform fraction of column An4 (GC parameters: 12.5 M x 0.25 mm WCOT; 60-280°C at 4°C/min.; 1.0 ml He/min)

b. TIC of starting material on same GC column, different conditions. (Parameters: 180-300°C at 8°C/min, hold at 300°C for 5 min; 2.0 mo He/min.)

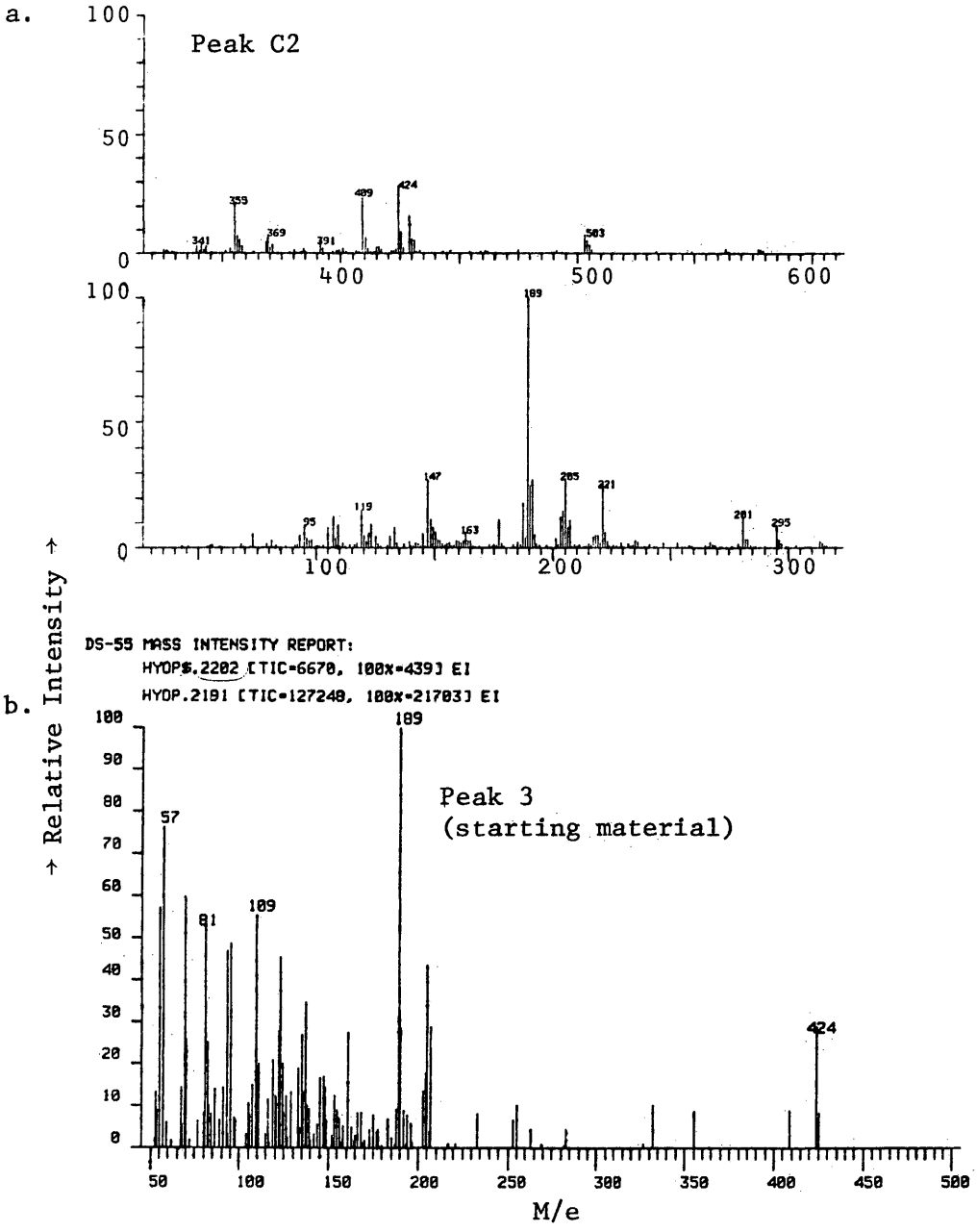
Figure 42

a.



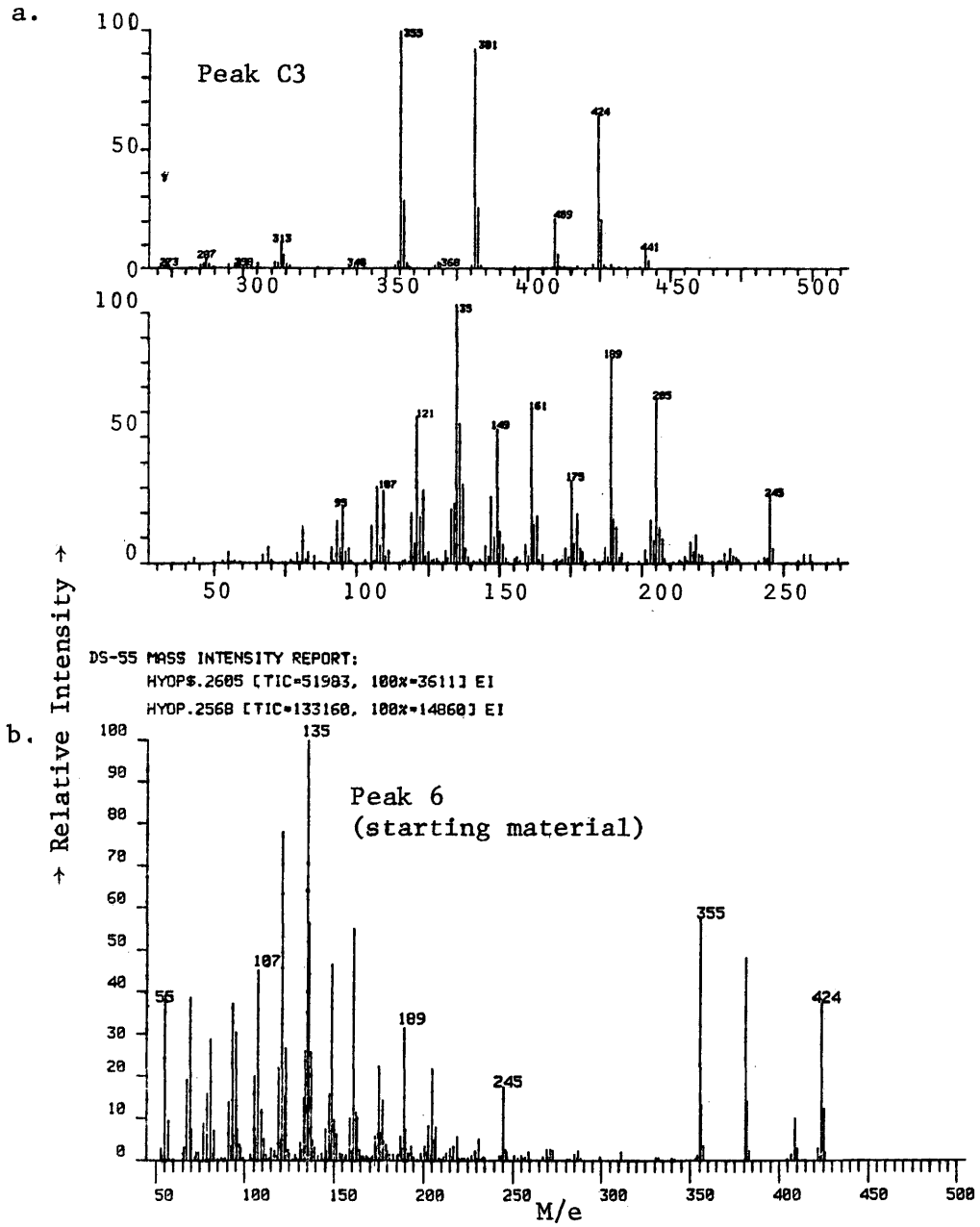
Mass spectrum of peak C1 (a) (column An4, chloroform fraction, compared with peak 1 of the starting material (b).

Figure 43



Mass spectrum of peak C2(a) (Column An4, chloroform fraction) compared with that of the third component of the starting material (b).

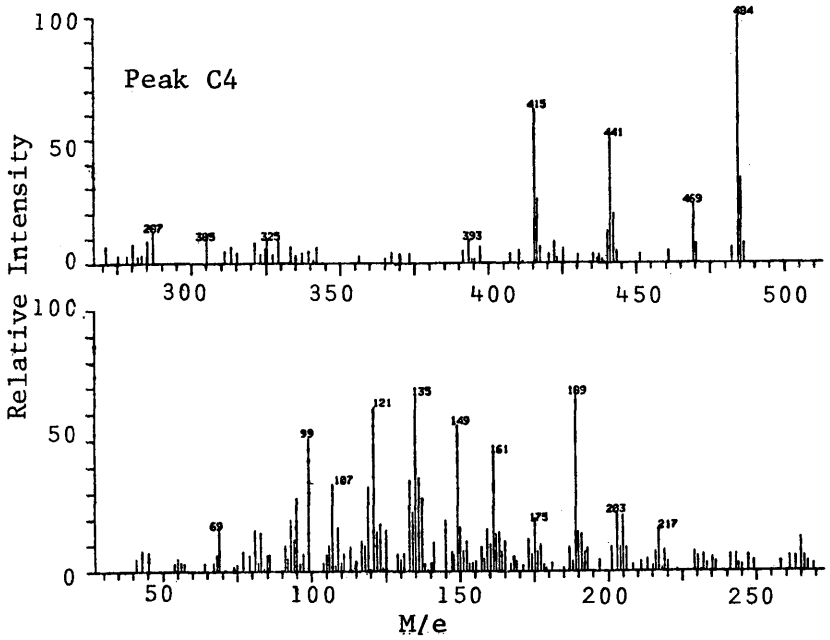
Figure 44



Mass spectrum of peak C3(a) (Column An4, chloroform fraction) compared with that of peak 6 of the starting material (b).

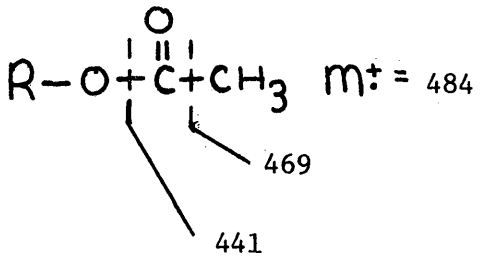
Figure 45

a.



Mass spectrum of peak C4 (column An4, chloroform fraction). This component was not detected in the starting material.

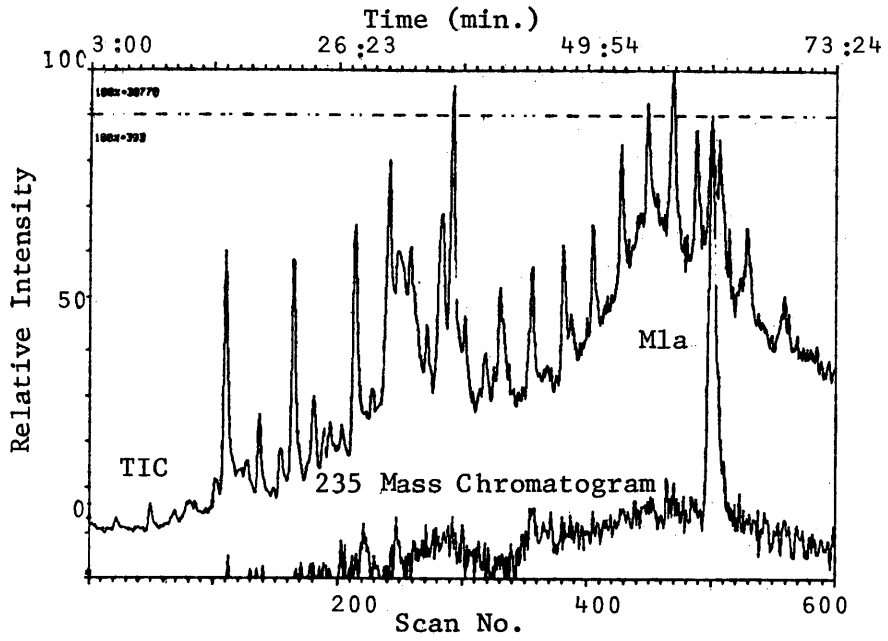
b.



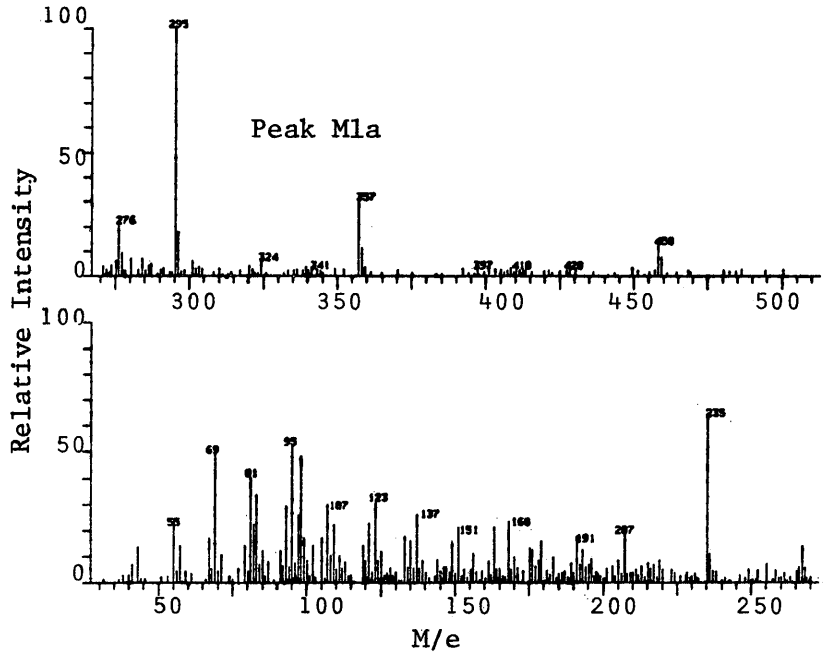
R = hopanoid isomer
possibly related to component 6

Figure 46

a.



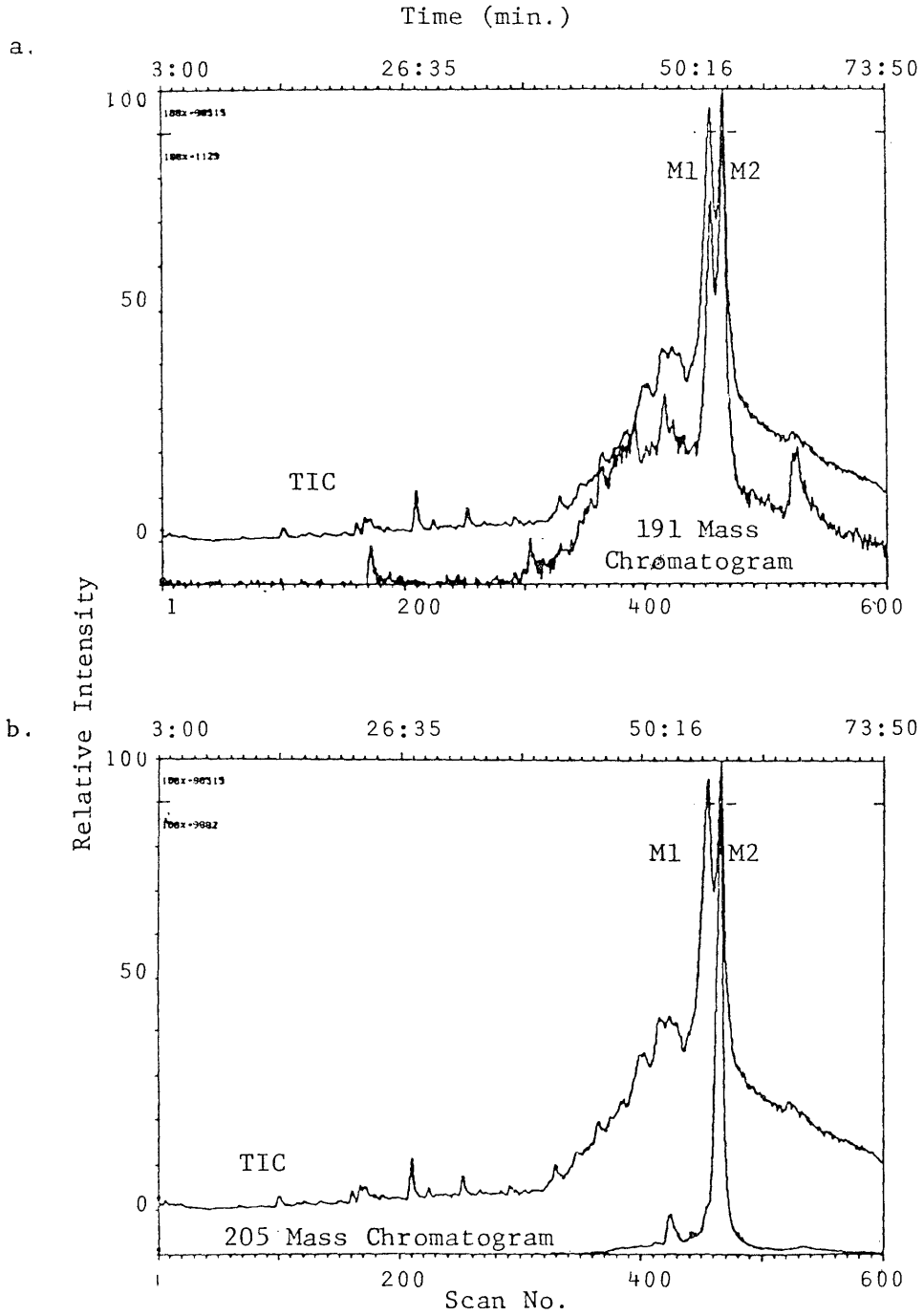
b.



a. TIC and 235 mass chromatogram of methyl esters of column A3. (GC parameters; 12.5M x 0.25 mm SE-52 WCOT; 60-300°C at 4°C/min.; 1.0 ml He/min.)

b. Mass spectrum of peak M1a.

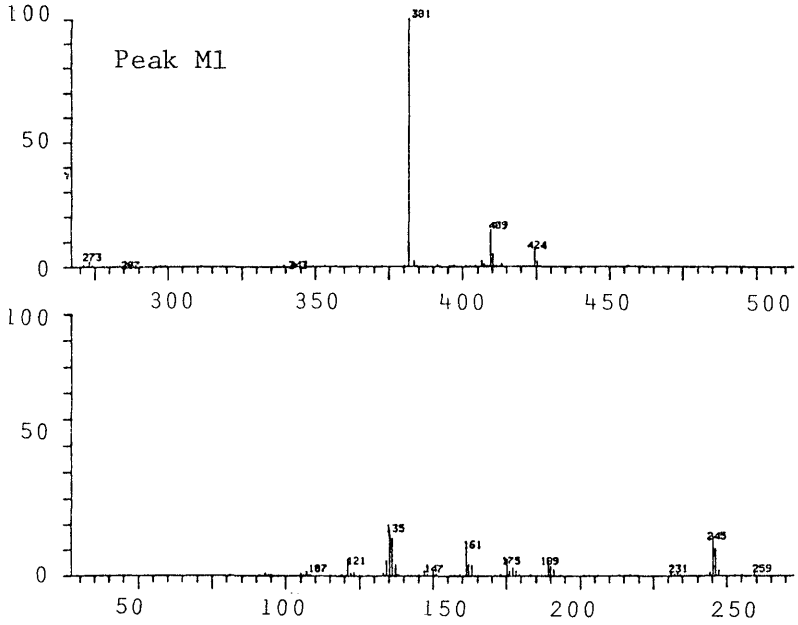
Figure 47



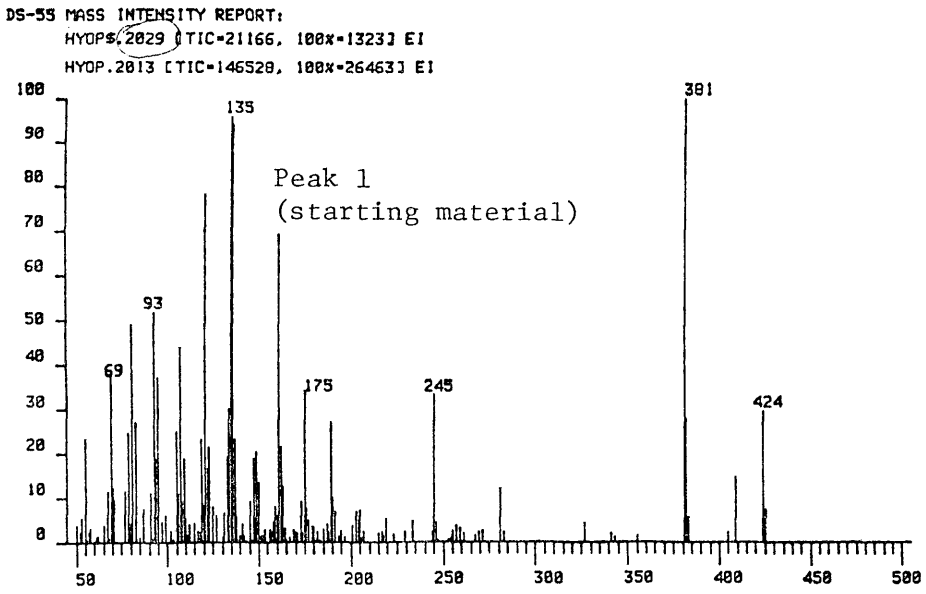
TIC and mass chromatograms of the methyl ester fraction of columns An3, (a) TIC and 191 mass chromatogram, (b) TIC, and 205 mass chromatogram. (GC parameters: 12.5 M x 0.25 mm SE-52 WCOT; 60-300°C at 4°C/min; 1.0 ml He/min.)

Figure 48

a.

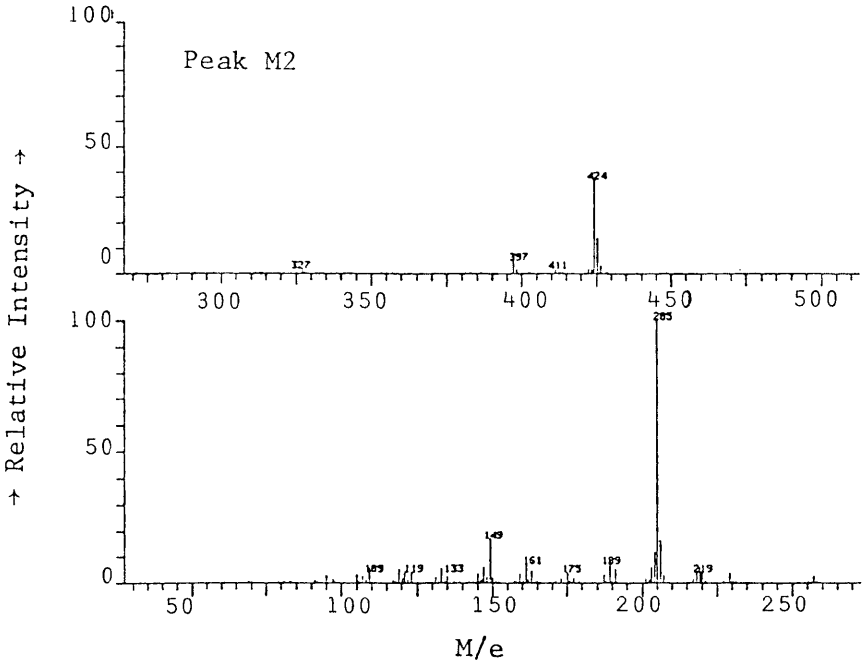


b.



Mass spectrum of peak M1(a) (column An3, methyl esters) compared with that of the first component of the starting material (b).

Figure 49



Mass spectrum of peak M2 (column An3, methyl esters). This peak could be related to the starting material, however the differences in intensities do not allow us to match it with any one particular starting compound. It is reasoned to be the 17∝(H) epimer of 17β(H),21β(H)-hydroxyhopanone.

Figure 50

DISCUSSION

Analysis of the Starting Material Isolated from Dammar Resin

Thin-layer chromatography of the starting material showed two components. These components were then resolved by liquid column chromatograph (see Experimental, p. 22).

Column chromatography (previously described) was employed to resolve the two components of the material isolated from Dammar resin. The boundary zone between the two components was found by use of thin-layer chromatography of samples from individual fractions. Infrared spectrophotometry was used to indicate the position of hydroxyhopanone relative to the boundary. Combined gas chromatography-mass spectrometry was used to give positive identification of the hydroxyhopanone.

Thin-layer chromatography indicated that the first two fractions of each column chromatographic column contained only one component, and that the benzene-chloroform fractions contained only the slower component. IR of the faster eluting material showed neither alcohol nor ketone absorbances at 3600-3300 cm^{-1} and 1700 cm^{-1} respectively (Figure 11a) IR of the second component showed both of these absorbances (Figure 11b). This indicated that hydroxyhopanone must be in the second component.

Gas Chromatography and Combined Gas Chromatography-Mass Spectrometry of the Dammar Resin Products*

Figure 12 is a gas chromatogram of the material believed to be hydroxyhopanone. The column used was a 30 M x 25 mm SE-52 glass capillary. The experimental conditions are included in the caption. The material appears to contain six compounds represented by peaks 1-6. Using peak height as a measure of concentration it was found that components 1-4 made up 5% of the mixture and that the other 95% of the material comprised 5 and 6 in a 1:1.3 ratio.

The material was next analyzed by GC-MS. Figure 13 shows the total ion current (TIC) of the GC-MS experiment. Unfortunately, a different type of column was used (a 20 m x 0.25 mm SE-54 glass capillary) and the resolution between the major components, peaks 5 and 6, was poor.

Figures 14a and 15 are the mass spectra of components 5 and 6 of the TIC. The spectra are similar in that they both have the same mass fragments.

---

*The data discussed in this section are from analyses performed by Dr. Denis Miiller of Phillips Petroleum Company, Bartlesville, Oklahoma.

The parent peak of both of these isomers is found at M/e 424 and represents hydroxyhopanone after the loss of water. Both components also have the other diagnostic peaks for hydroxyhopanone at M/e: 409, 205, 189, and 149. However, they differ in the relative intensities of these and other mass fragments.

A comparison of these two mass spectra with mass spectrum of authentic hop-22(29)-en-3-one obtained by computer search (Figure 14b) indicates that component 5 gives the best match and is probably hydroxyhopanone.

Note that in neither of the spectra of components 5 and 6 is the molecular ion for hydroxyhopanone (M/e 442) present. This is due to the rapid loss of water from alcohols upon ionization forming the $(M-18)^{+}$ mass fragment, mass 424 for hydroxyhopanone. The molecular ion is observed when the mixture is analyzed by direct probe insertion (Figure 16)

Component 6 is believed to be a structural isomer of hydroxyhopanone. Its identity is unknown. The four minor constituents are thought to also be keto-alcohols related to 5 and 6.

Bacterial Growth: General

The measurement of the relative growth of the bacteria was performed to determine the level of growth in both the

"living" columns (An1, An2, An3, and An4 of the anaerobic experiments; and A1, A2, A3, and A4 of the aerobic experiments) and the poisoned columns (An5, An6, and An7 of the anerobic experiments; and A5, A6, and A7 of the aerobic experiments)

Anaerobic Experiments: Bacterial Growth

The differences in bacterial growth above the water-sediment interface and between the "living" columns and their poisoned analogues can be seen in Figures 17a through 17c. Note that there are differences in the shapes of the growth curves among the "living" columns themselves. The curves of the poisoned columns also vary from one to the other, but these differences are easily discernable.

Figure 17a shows a typical growth curve representing the bacterial multiplication in column An1. This column contained only sediment and sea water. The bacteria in the water reached a plateau in population between 10^7 and 10^8 colony-forming units per ml (CFU/ml)

Column An2 (Figure 17b) showed an unexpected growth pattern; rapid multiplication during the first few days of incubation, reaching nearly 10^7 CFU/ml, followed by a rapid decline in the microbial population to about 2 x 10^4 CFU/ml. Columns An3 and An4 (Figure 17c) experienced a retardation in microbial growth. Since columns An3 and An4 contained yeast extract and glucose it was expected that they would show greater numbers of colony-forming units per milliliter than column An1. As shown in Appendix A, the presence of dimethylformamide is believed to have inhibited the growth of the bacteria in these columns.

Each of the poisoned columns did exhibit some bacterial multiplication at some time during the incubation period. The growth was especially high in columns An5 and An6 (Figures 17a and b) The reason the curves do not go below the 10^3 CFU/ml mark is that the samples were diluted by a factor of 100 of which 0.1 ml was plated; hence 10^3 CFU/ml is a maximum value.

The bacterial populations 3 to 4 cm below the water-sediment interface were more prolific than those of the water just above it (Figures 18a-c) This difference in the density of bacterial colonies between the water and the sediment is especially dramatic in the poison control columns. In the water of these columns the populations were

typically 10^3 CFU/ml or less, whereas in the sediment the numbers of colonies per ml ranged from 10^5 to 10^8, depending on the column.

It is thought that a number of factors caused the poison, $HgCl_2$, to be ineffective: (1) the high surface area of the fine-grained sediment, which gave the bacteria an advantage as the Hg^{++} ions were not available to all these surfaces; (2) the complexation of the Hg^{++} ions by hydroxyl groups; (3) in anaerobic columns the formation of HgS precipitates during the production of H_2S.

Anaerobic Experiments: pH of the Water

All pH values for this set of experiments remained in the pH range found for most natural waters, i.e., 4-9. The pH behavior of the "living" columns varied from column to column, while the poisoned columns behaved similarly with respect to pH, generally remaining near 7.

Figure 19a shows that column An5 generally had a lower pH than that of column An1. This difference is thought to be due to the addition of $HgCl_2$ to column An5 as a poison. When mercuric chloride dissociates the mercuric ion complexes with the hydroxyl group of water and protons are produced, thereby lowering the pH of the water. The pH in column An5 did trend upward during the experimental period

to merge with that of An1. It is thought that the precipitation of HgS caused the reversal of the reaction described above.

Columns An2 and An6 (Figure 19b) show nearly the mirror image of columns An1 and An5 (Figure 19a) This effect may be explained by the fact that column An2 showed a decline curve with respect to bacterial growth in the water, while An1 experienced a growth curve.

Columns An3, An4, and the poisoned analogue An7 behaved more like columns An1 and An5 with respect to their trends in pH (Figure 19c). The waters in columns An3 and An4 were more alkaline than that of An7, and they did tend to merge during the experimental period. The drop in pH between days 10 and 27 may be due to the erratic type of growth which these columns experienced.

Among the poison controls, An5 has the highest pH values throughout, while An6 and An7 behave more similarly. This makes sense as An6 and An7 had nearly identical contents.

Anaerobic Experiments: Eh

Eh of the water and sediment was measured to qualitatively describe the redox conditions in each of the columns. Highly positive potentials were equated with a high oxygen content. Negative potentials were equated with conditions

favoring anaerobic growth. These assumptions were based on the observations of workers in the field of natural environments (Zobell, 1946; Baas Becking et al., 1960; and Morris and Stumm, 1967)

All columns showed more positive Eh values in the water above the sediment than in the sediments. Eh decreased with depth into the sediments. (See Figure 20a-g.)

The Eh in the water of column An1 never went below 20 mV (Figure 20a). While the oxygen content in the water may have become somewhat depleted, the mere actions of getting into and out of our columns may have been enough to reintroduce oxygen into the water. The water was always in communication with the atmosphere to some degree. The water layer was essentially used as a barrier in an attempt to maintain the Eh in the sediment of the "living" columns as reducing as possible.

As can be seen in Figure 20a, the Eh measured at a depth of 3 to 4 cm into the sediment decreased with time to -250 mV during the first 30 to 40 days. The Eh at the bottom of the column also showed this decrease. The subsequent rise in Eh after 40 days remains unexplained, but probably points to problems with our experimental procedure. A slight H_2S odor was detected from the sediments in this column.

The Eh of the water and the uppermost 3-4 cm of sediment of column An2 dropped dramatically during the first five days of the experiment, from 350 and 300 mV to -350 and -400 mV respectively (Figure 20b). There was fluctuation in the values from measurements made in the water. The Eh in the sediments generally leveled out at about -250 mV from Day 20 onward. The sediments in this column did reach and maintain an anoxic state. These sediments had a very strong H_2S odor.

In column An3 the water remained highly oxidizing with the Eh generally remaining above 350 mV (Figure 20c) In the above 3-4 cm of sediment the Eh slowly dropped during the first 30 days, climbed, and then dropped dramatically during the last 20 days. The Eh at the bottom of the column decreased during the time it was measured, reaching a final low value of -350 mV. The sediments in this column also had a strong H_2S odor.

The Eh behavior of column An4 was not expected (Figure 20d) At all depths the Eh remained in the oxidizing realm until sometime during the last ten days. This was surprising as the bacterial growth in the water and sediments of this column was very similar to that of column An3, and the sediment did have a slight H_2S odor, although less potent than that of An3.

In column An5, the poison control analogue of An1, the Eh at all three locations was expected to remain oxidizing (Figure 20e). The Eh of the water did remain oxidizing; usually greater than +350 mV. While the Eh in the uppermost 3-4 cm of sediment remained above +350 mV for the first 30 days of the experiment, it did decrease to low values (-250 to -180 mV) during the last 23 days. This is not surprising since it was found that the bacteria at this depth did multiply. Behavior of the redox potential at the bottom of the column, while at first erratic, did settle down to values between -224 and -175 mV during the last 23 days of the experiment. The sediments in this column had a slight H_2S odor.

The Eh of the water in the poisoned column An6 (Figure 20f) remained oxic throughout the incubation period. The uppermost 3-4 cm of sediment also remained highly oxidizing (above +350 mV) during the first 40 days of the experiment. Note that at the bottom of the column the Eh was always negative (between -200 and -300 mV) The values at the bottom of the column are very near those of column An2. (Column An6 is the poisoned analogue of An2.) The smell of H_2S was also detected from the sediments of this column.

Column An7 behaved in the way that all the poison control columns should have behaved. The Eh at all depths

remained oxidizing (above 200 mV). The Eh does, however, remain stratified in relation to depth, with the deeper locations having the lower values. Column An7 also had a faint H_2S odor.

Aerobic Experiments: Bacterial Growth

Column A1 (Figure 21a) showed rapid multiplication of bacteria in the first two days of incubation, followed by a decline in the population, which then leveled off at about 10^5 CFU/ml.

Column A5 (Figure 21a), the poison control analogue of A1, experienced a small increase in population of bacteria during the first two days after which the numbers of colony-forming units per ml was never greater than 10^3.

Growth in column A2 (Figure 21b) was extremely erratic. This could be due to the presence of dimethylformamide in this column. As shown in Appendix A, DMF does appear to inhibit bacterial multiplication.

The poisoned analogue of A2, A6, showed no multiplication above 10^3 CFU/ml. The actual numbers may have been less; however the samples were always diluted by a factor of 100, of which 0.1 ml was plated; hence we can only say that the numbers of colony-forming units was equal to or less than 10^3/ml (the same is true for A5 and A7)

Column A3 also experienced erratic growth; again this may be due to the presence of DMF (Figure 21c) On the other hand the bacteria in column A4, while inhibited during the first ten days of the experiment, showed intense multiplication afterward, the population on the final day of the experiment being in excess of 10^9 CFU/ml (Figure 21c)

As with the other poisoned columns, A7 exhibited no bacterial populations in excess of 10^3 CFU/ml. Measurements were not made on days 50 and 60 due to evaporation (Figure 21c)

Overall it can be said that the bacterial multiplication in the "living" columns was significantly greater than that of their poisoned analogues. However, it cannot be stated that the bacteria were completely poisoned, but rather only that their growth remained below a certain level.

Aerobic Experiments: pH

At the outset of each experiment the waters of the poisoned columns all had lower pH values (5.7-5.8) than their "living" counterparts (7.0-7.4). This is due to the acidifying effect of $HgCl_2$ as explained previously on page 72.

While the pH values in the waters of columns A1 and A5 remained divorced throughout the experimental period, those of others do not (Figure 22) After Day 10 columns A2 and A6 behaved quite similarly with respect to pH. In columns A3 and A4 the pH trends toward lower values. In column A4,

however, the pH increases dramatically after 30 days. The rise in pH in column A4 may be linked to the sudden spurt in bacterial growth.

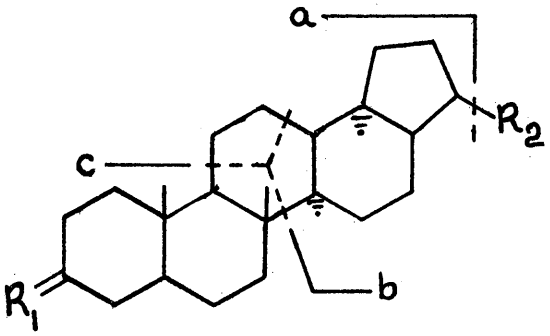
Gas Chromatography-Mass Spectrometry of the Sediment Extracts: General Comments

The organic extracts of the blank sediments of the anoxic columns An1 and An2, and those of oxic columns A1 and A2 were analyzed to ensure that any alteration products found in An3, An4, and An7; and A3, A4, and A7 were not indigenous to the sediment Because of the failure of the poison to work in the anoxic columns, An5 and An6 (poison analogs of An1 and An2, respectively) were not analyzed. Columns A5 and A6 (poisoned analogs of A1 and A2, respectively) were also not analyzed as it was thought that these columns would show no significant differences from the "living" columns.

Heptane and Benzene Fractions

The heptane and benzene fractions of the oxic columns A3 and A4, and the anoxic columns An4 and An7 contained alteration products of the starting materials All of these products were thought to result from dehydration of three of the starting materials These products were found by scanning for the position of the 189 and 191 mass fragments produced by cleavage of hopanes and hopanoids shown in Figure 51

The benzene fraction of column A4 contained only one compound, peak B2a (Figure 40) which appeared to be related



a = 369: R_1 = H(2)

b = 148 + R_2: R_2 = C_3H_5; b = 189

c = 191: R_1 = H(2)

205: R_1 = 0

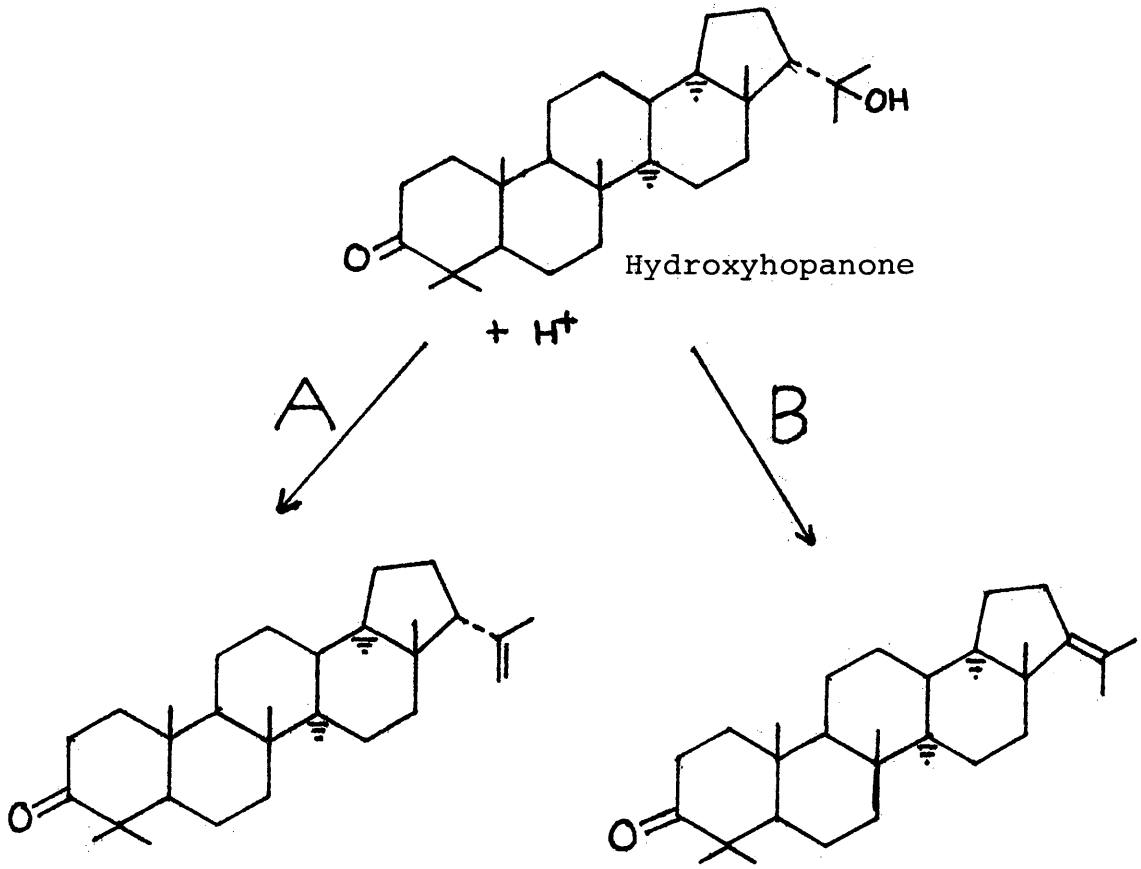
Major Cleavages of Hopanes, Hopenes, and Hopanoids, Showing the Major Fragments Produced

Figure 51

to the fifth component of the starting material, hydroxyhopanone (Figure 41) This compound is an olefinic-ketone related to hydroxyhopanone by dehydration. Because of the absence of this product and other dehydration products from the poison control column, A7, it is believed that this alteration takes place biologically. If the alteration had taken place abiologically it would be expected that there would be two products; the thermodynamic or Zaitsev product and the kinetic product. A general mechanism for dehydration of hydroxyhopanone is given in Figure 52. Path A (Figure 52) is thought to be analogous to the biological pathway. This was the only column in which an alteration product of hydroxyhopanone was identified.

In the benzene fraction of column A3 a dehydration product of the first component of the starting material was found, peak Bla (Figures 38 and 39). The structure of the first component of the starting material and its apparent dehydration product are unknown. However, it is felt that this dehydration product is produced by a mechanism analogous to that previously discussed (above).

Dehydration products of the sixth component of the starting material were found in the heptane exfraction of columns An4 (peak H4, Figures 26 and 28); An7 (peak H9, Figures 29 and 33); and A4 (peak Hla, Figures 34 and 35)



Hop-22(29)-en-3-one
Biological Product
(Kinetic Product)

Hop-21(22)-en-3-one
Zaitsev Product
(Thermodynamic Product)

Two products expected from acid catalyzed dehydration of hydroxyhopanone. Pathway A is assumed to be analogous to the enzymatic pathway of living organisms.

Figure 52

This product was also found in the benzene fraction of column An7 (peak B1; Figures 36 and 37). Again, dehydration of this compound is thought to occur analogously to that of hydroxyhopanone previously discussed on page 112 However, because of the failure of the poison to work in the anoxic column An7, biological alterations products could not be distinguished from abiological products in the anoxic experiments.

Several other compounds of interest were found in the heptane fractions of colunns An4 and An7. Peak H3 (Figure 26) may be related to peak H1 of column An1 (Figure 23) The mass spectrum of peak H3 (Figure 27) has a similar M/e 189 to 191 ratio as that of peak H1 (Figure 24) This compound is indigenous to the sediment. Other compounds in the heptane fraction of column An7 were peaks H5-H8 (Figures 29-32) Peaks H5 and H6, although not found in the blank sediment, are difficult to relate to the starting materials because of the large differences between their mass spectra and those of the starting compounds. Peak H7 yields a mass spectrum very much like that of peak H2 (column An1) and diploptene (Figure 25 a and b) Peaks H2 and H7 are believed to be diploptene, which is indigenous to the sediment. The mass spectrum of peak H8 (Column An7, Figure 32) has several peaks which relate it to the starting materials at

M/e 424, 409, 355, 205, and 189. It is not known which of the starting materials this compound represents. The mass peaks at M/e 429 and 449 should be ignored as the losses required from these ions to produce the 424 fragment are impossible. These fragments are probably ions of other compounds coeluting under peak H8. The molecular ion of the compound of interest is at M/e 424.

It should be noted that the highly reducing sediment of column An3 produced no dehydration products of any of the starting materials, whereas columns An4 and An7, which both had relatively more positive, oxidizing E_h values, did produce dehydration products. This may have significance regarding short term diagenesis of hopanols, in oxidizing vs reducing depositional environments. Preservation of the hydroxyl function is supported by the findings of Nishimura (1977). Nishimura reported an increase in stanol concentration with depth in anoxic sediments. However, Welte and Waples (1973) give evidence which supports dehydration of n-fatty alcohols under reducing conditions as a route for the production of the even n-alkanes of sediments.

Chloroform Fractions

The behavior of the starting material during the purification step (p. 22) indicated that any unaltered starting material was likely to be present in the chloroform fractions of the sediment extracts.

The TICs and M/e 205 traces of the chloroform fractions of columns An3, An4, An7, A3, A4, and A7 are all nearly identical. Only that of column An4 is shown and compared with the TIC of the starting material analyzed on the same GC column but under different conditions (Figure 42, see caption). The sediment extracts are essentially made up of starting material. The peaks to the left of C1 are components of the sediment. Peaks C1, C2, and C3 yield mass spectra identical to those of the first, third, and sixth components of the starting material from Figure 13 (Figures 37, 38, and 39, respectively). As can be seen, the resolution in Figure 13 was far superior to that achieved in Figure 42 (a and b) Peaks 2 and 4 of Figure 13 are not visible in Figure 42 (a and b). Peaks 5 and 6 of Figure 13 have coeluted to give peak C3 of Figure 42 (a and b) Peak C4 of column An4 is apparently a new compound. It was not found in the blank sediments, nor in the starting material. The mass spectrum of C4 (Figure 45) indicated that it may be related to the sixth component of the starting material

by comparison of the fragmentation patterns between M/e 90 and 190. The molecular formula of C4 was found to be $C_{32}H_{52}O_3$ by dual beam mass spectral analysis. The compound is assumed to be an acetate (Figure 46b) The fragments produced at the high mass end indicate this to be very probable. Acetylation of the hydroxyl oxygen of any of the starting materials yields the correct molecular weight.

It was at first assumed that peak C4 was produced biologically due to its higher molecular weight relative to the components of the starting material However, its presence in the sediments of column A7 (the poisoned analogue of A3 and A4) sheds doubt on this hypothesis. Column A7 had very low microbial counts during the first 40 days, and the counts are assumed to have remained low after 40 days because of evaporation, which would have concentrated the poison further. Perhaps this compound was actually present in the starting material and not detected until this time. The origin of this compound is therefore in doubt.

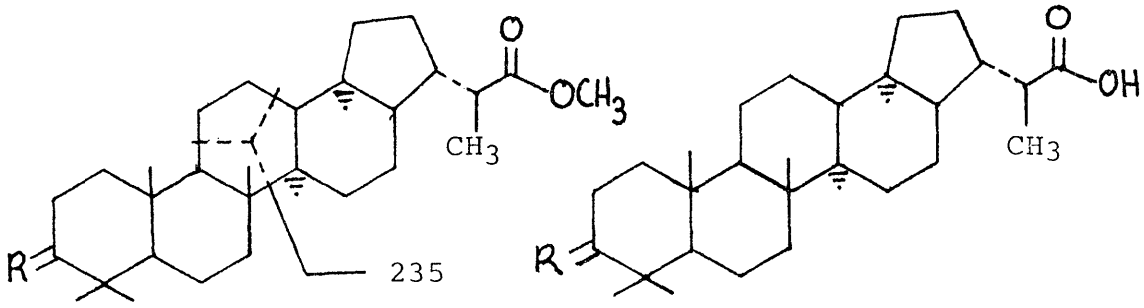
Columns An1 and An2 contained no components which could be related to those of the starting material No TICs or mass spectra are shown here for columns An1 and An2.

Methyl Esters

The mass chromatogram of the 235 ion which forms from the fragmentation of the methyl ester shown in Figure 53 was thought to be the key to the presence of the carboxylic acid of this methyl ester.

The methyl esters of oxic columns A3, A4, and A7 all showed one major peak in their M/e 235 mass chromatograms, while columns A1 and A2 (blanks) did not. (Only the TIC and M/e 235 mass chromatogram of column A3 is shown, Figure 47a). Note the major peak, M1a, of the mass chromatogram. The mass spectrum of peak M1a (Figure 47b) does not seem to relate it to any of the starting materials. The molecular formula was found to be $C_{29}H_{46}O_4$ by dual beam analysis; the structure is unknown. However, the high abundance of this ion in the fractions from columns A3, A4, and A7, and its complete absence from blank sediments points toward some sort of alteration of one of the starting materials. The fact that this product was found in the poisoned column indicates that it is an abiological alteration product.

The 235 mass chromatogram of the methyl ester fractions of the anoxic columns showed no clear features. It was decided to use the ions (191 and 205) which result from normal hopane type cleavages (Figure 51) to try to locate products of the anoxic columns (Figure 48) The mass spectra of the



Methyl Ester Acid

R = O, H(2)

Proposed carboxylic acid (right) of the saponifiable fraction of the sediment extracts. The methyl ester of the proposed acid is shown at left. One of the major fragments expected is also shown above.

Figure 53

two major peaks, M1 and M2 (Figures 49 and 50) of column An3 (An4 and An7 not shown, but identical) show that both appear to be related to the starting material Neither M1 nor M2 is a methyl ester. The ion at M/e 424 in each spectra indicates that M1 and M2 are the hydroxy-ketones or the dehydration products of the starting materials. Their presence indicates that the saponifiable-nonsaponifiable separation was not complete. It is most likely that both compounds which remained in the saponifiable fraction were keto-alcohols, although they may have dehydrated during the esterification procedure.

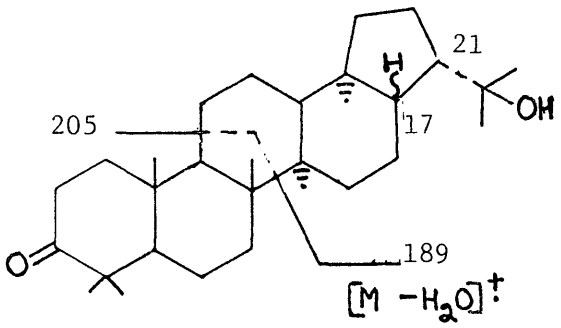
Peak M1 appears to be related to the first component of the starting material by mass spectral comparison (Figure 42) It is probably the starting material itself, or a dehydration product of it.

Peak M2 is more interesting. Because of the different mass spectrum it exhibits, the base peak at M/e 205 and the minor 189 mass fragment, it cannot be related directly to any particular starting component. The inversion in the intensities of these fragments (205>>189) contrasted with the intensities of the 189 and 205 fragments of the starting materials (189>>205) points toward epimerization at C-17 Wardroper (1977) states that hopanes of the 17β(H),21β(H) type always yield a "148 + R" mass peak which is more

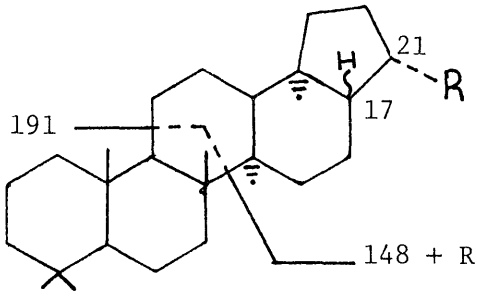
intense than the 191 mass peak. On the other hand, 17α(H), 21β(H) hopanes show the reverse. The analogy which is drawn here is that in hydroxyhopanone (or Hop-22(29)-en-3-one) the 205 fragment is to the 189 fragment as the 191 fragment is to the 148 + R fragment of hydrocarbon hopanes. (See Figure 54 for a pictorial analogy).

Peak M2 may be the 17α(H),21β(H) epimer of 17β(H),21β(H)-hydroxyhopanone (or Hop-22(29)-en-3-one), if the analogy discussed is valid; or it could also be an epimer of one of the other starting materials. It is hypothesized that this epimer was formed under the highly acidic conditions of the ester-forming reaction mixture. The mechanism is shown in Figure 55.

Tables 4 and 5 summarize the findings of the incubation experiments.



Hydroxyhopanone



Hopanes

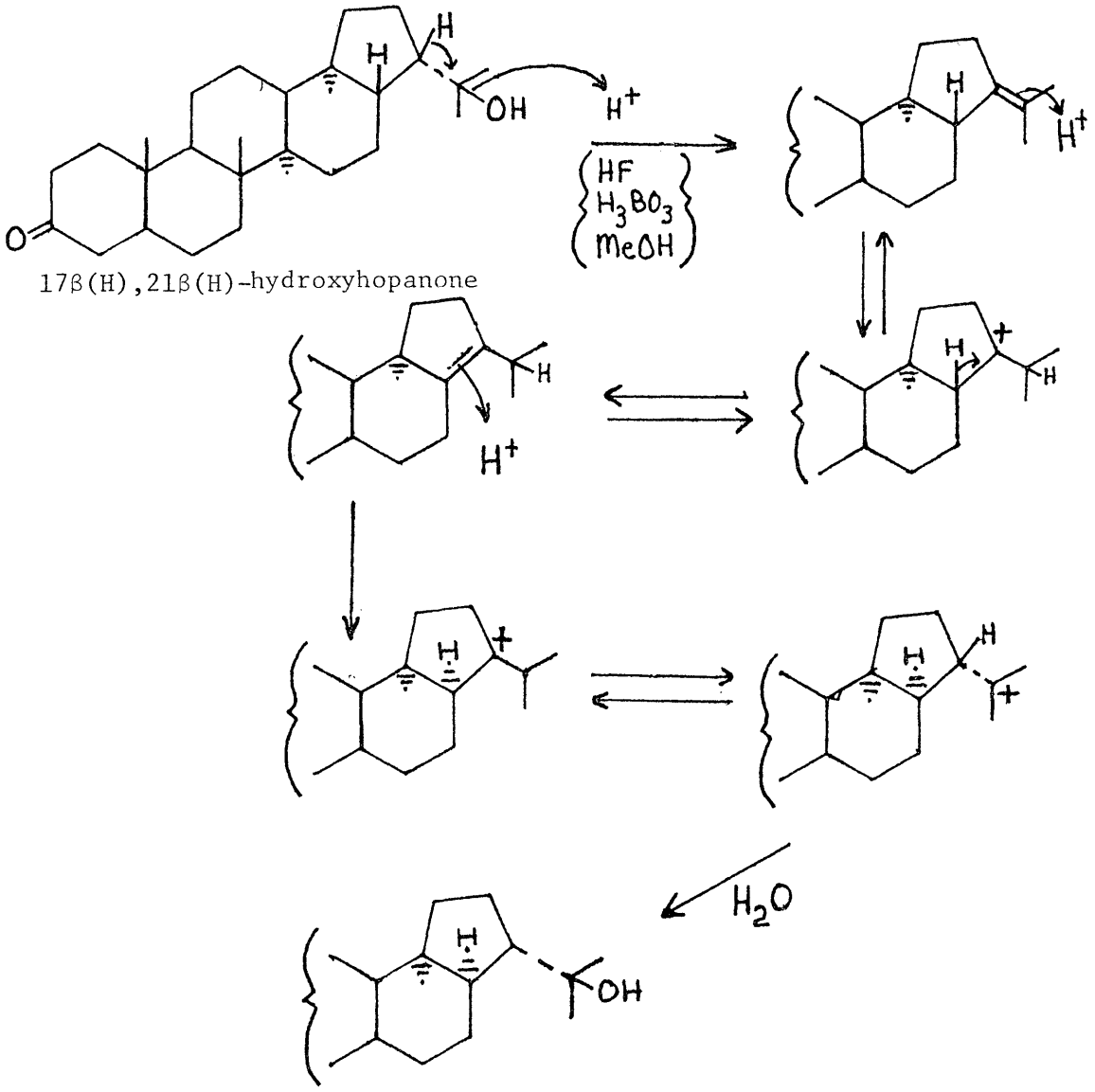
When:

M/e:189>>205⟹17β(H),21β(H); 148+R>>191⟹17β(h),21β(H)

M/e:189<<205⟹17α(h),21β(H); 148+R<<191⟹17α(H),21β(H)

Proposed fragmentation of hydroxyhopanone (left) yielding fragments analogous to those of hopanes (right) (Wardroper et al, 1977; Van Dorsellaer, 1974 and Ensminger et al,1974). The relative intensities of these fragments is dependent on the configuration about C-17

Figure 54



17β(H),21β(H)-hydroxyhopanone

17α(H),21β(H)-hydroxyhopanone

Hypothesized Acid Catalyzed Epimerization of Hydroxyhopanone Producing 17α(H),21β(H)-hydroxyhopanone

Figure 55

TABLE 4

Presence of Alteration Products
Related to the Starting Materials Anoxic Columns

Column	Conditions	Fractions from Column Chromatography			
		Heptane	Benzene	Chloroform	Methyl Esters
An1	Negative E_h	H1-diploptene H2- ?	———	———	———
An2	Negative E_h	———	———	———	———
An3	Negative E_h	———	———	Starting material + Compound C4 (Origin?)	M1-Related to one of starting material? M2-epimer of hydroxyhopanone? Both from poor saponifiable-nonsaponifiable separation
An4	Positive E_h	H4-Dehydration product of 6 of starting material	———	"	"
An7	Positive E_h	H9- "	B1 (same as H9)	"	"

TABLE 5

Presence of Compounds
Related to Starting Materials – Oxic Columns

Column	Days	Fractions from Column Chromatography			
		Heptane	Benzene	Chloroform	Methyl Esters
A1	60	———	———	———	———
A2	60	———	———	———	———
A3	30	———	B1a Dehydration product of first component of starting material	Starting material + C4 (Origin?)	M1a Not like starting materials
A4	60	H1a-Dehydration product of 6th component of starting material	B2a-Dehydration product of hydroxyhopanone	"	"
A7	60	———	———	"	"

Conclusions

The only alteration product of hydroxyhopanone which was found was a dehydration product; Hop-22(29)-en-3-one. This compound was found in the 60-day "living" oxic column (A4) and is believed to be biologically produced.

Dehydration products of the first and sixth components of the starting material were also found. Neither the structures of these starting compounds nor their products are known. Dehydration of the first component occurred in only the 30-day "living" oxic column. The dehydration product of the sixth component was found in the 60-day "living" oxic column, the 60-day "living" anoxic column, and the 60-day poisoned anoxic column. In the oxic columns the dehydration products are biological due to their absence in the poisoned oxic column. However, because of the failure of the poison to work in the anoxic poisoned control, biological and abiological alterations cannot be distinguished.

No dehydration products were found under the highly reducing conditions of column An3, although they were found under the oxidizing conditions of columns An4, An7 A3, and A4 This indicates that alcohols do not rapidly dehydrate under reducing conditions and are preserved for at least a short time ($\sim$ 60 days) after deposition.

In all the columns which contained starting material a new compound was found in the chloroform fractions. The molecular weight of the compound was 484. Its identity is unknown, but its higher molecular weight points toward biological production. However, the fact that this component was found in an apparently working poison control sheds doubt on the origin of this compound.

In the methyl ester fractions of the oxic columns another new compound was found, but its mass spectrum did not allow us to relate it to the starting materials. The presence of this compound in the poisoned column points toward abiological alteration of one of the starting materials

Because of incomplete separation of saponifiables from non-saponifiables two compounds were found in the methyl ester fractions of the anoxic columns that were not methyl esters. One is believed to be the first component of the starting material The other is reasoned by analogy to be a 17α(H) epimer of one of the starting materials, possibly hydroxyhopanone.

The presence of 17α(H),18α(H) 21β(H)-28,30 bisnorhopane (28) was not detected in our sediments, indicating that this compound is not formed from hydroxyhopanone during the early stages of diagenesis No precursor to this

compound has been found to date.

An effort has been made to study the diagenetic alteration products of hydroxyhopanone, a hopanoid of terrestrial origin. Incubation of hydroxyhopanone in two different model depositional settings, an oxic stream sediment and an anoxic marine sediment, for 60 days resulted in only one alteration product; Hop-22(29)-en-3-one. This was found in only the oxic stream sediment. This study has been an attempt to increase our understanding of the fate of natural products,during early diagenesis. In order to better understand the relationship between deposition environments and their organic matter, and to eventually associate source rocks and fossil fuel reservoirs with the organisms and depositional environments from which they originated, we must deduce the diagenetic and catagenetic changes which organic compounds may undergo. Hopanes which have been found in many land plants, and an ever increasing number of bacteria, accompanied by the fact that they are ubiquitous to sediments, source rocks, and crude oils, are likely candidates for this purpose. Laboratory experiments which attempt to model the natural environment are one means by which man may better comprehend the subtle chemical changes which hopanes and other compound types undergo between deposition and incorporation into fossil fuel reservoirs.

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APPENDIX A

Results and Discussion DMF Inhibition Tests

As growth in the anaerobic columns An2, An3, and An4 did not equal that of the blank column An1, it was postulated that the presence of dimethylformamide in these columns caused the low growth. As previously stated, growth in columns An2, An3, and An4 was expected to exceed that of column An1 because these columns contained yeast extract and glucose, while column An1 did not.

Slurries of marine sediment and sea water solutions were made up, bottled, and incubated for six days. The slurries made up in these experiments maintained the same relative percentages of nutrients, dimethylformamide and $HgCl_2$, as did the columns incubated in the major experiments. The different slurry mixes are shown in Table A-1

TABLE A-1

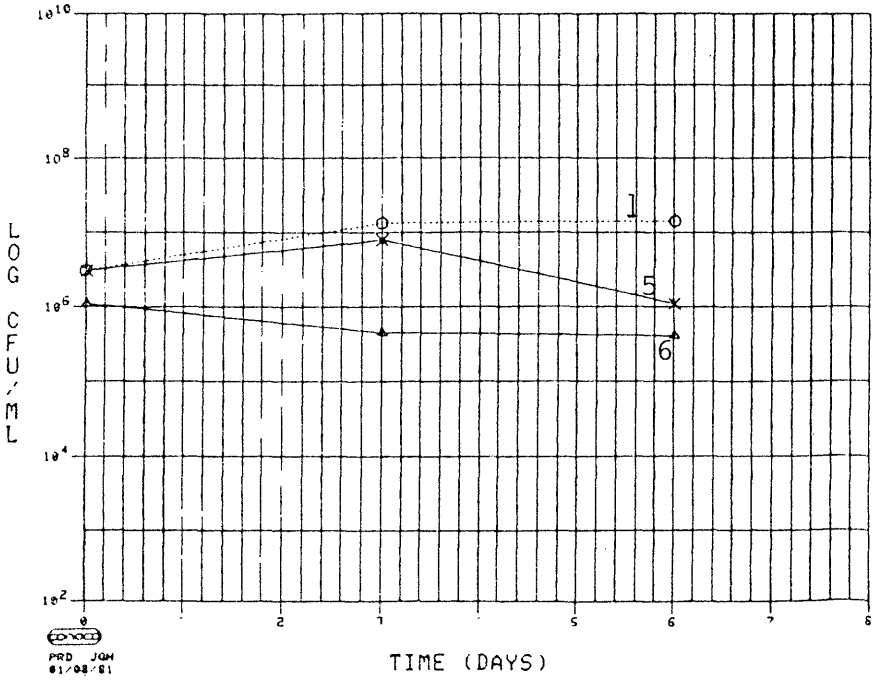
DMF Inhibition Tests: Bottle Contents

Bottle	Contents	Figure
1	Sea water and sediment	A-1
2	Sea water, sediment, nutrients	A-2
3	Sea water sediment, nutrients, & DMF	A-2
4	Sea water, sediments, nutrients, DMF, and $HgCl_2$	A-2
5	Sea water + sediment + DMF	A-1
6	Sea water + sediment + $HgCl_2$ + DMF	A-1

The plots shown in Figures A-1 and A-2 are of the log of the number of colony-forming units per ml versus time in days.

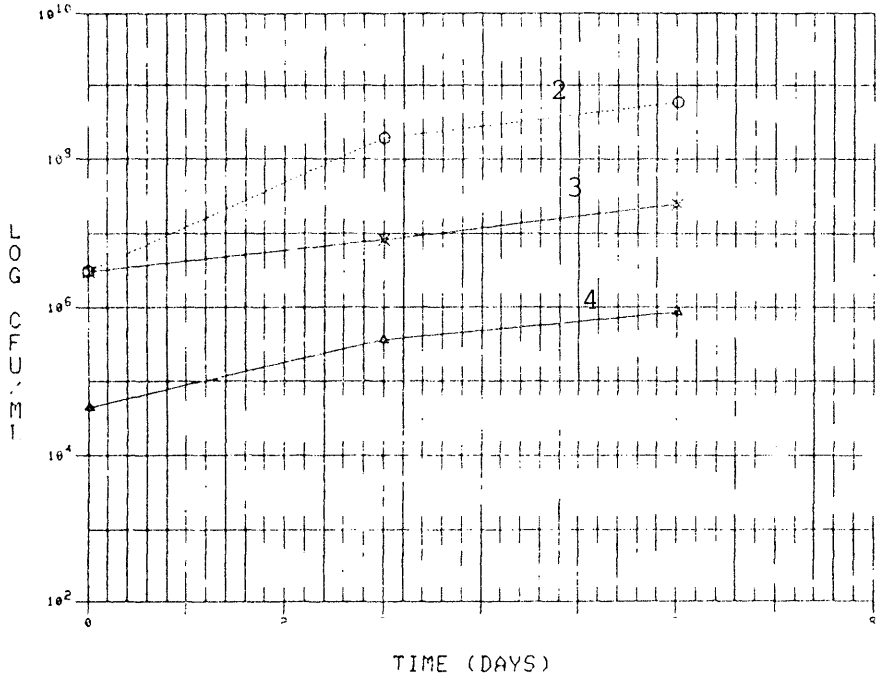
In Figure A-1 we see that bottle 1 had the most colony-forming units, that bottle 5 had fewer during the six days, and that bottle 6 had the fewest. This progression makes sense as one views Table A-1 again: bottle 1 was the blank; bottle 5 contained dimethylformamide and had fewer colonies; and bottle 6, which contained both dimethylformamide and $HgCl_2$, showed a negative growth curve.

Figure A-2 gives a very clear picture of the inhibition of growth caused by the presence of dimethylformamide. Bottle 2 showed the greatest growth and contained only nutrients, sediment, and sea water. Bottle 3, which contained dimethylformamide and nutrients, showed growth which was more than an order of magnitude less than that of bottle 2. Bottle 4, which contained $HgCl_2$, dimethylformamide, and nutrients, showed the least amount of bacterial multiplication, as was expected. All of these results lead to the conclusion that dimethylformamide does indeed inhibit bacterial growth.



Results of DMF inhibition tests for experiments 1, 5 and 6. See Table A-1 for the contents of each container CFU = Colony Forming Units.

Figure A-1



Results of DMF inhibition tests for experiments 2, 3 and 4. See Table A-] for the contents of each container. CFU = Colony Forming Units.

Figure A-2

APPENDIX B

WEIGHTS OF EXTRACTS AND FRACTIONS FROM COLUM CHROMATOGRAPHY

TABLE B-1

Weights of Extracts and Fractions

From Column Chromatography in mg

Column	Total Extract	Heptane Fraction	Benzene Fraction	Chloroform Fraction	Methanol Fraction	Methyl Esters
An1	203.85	5.92	0.71	15.38	8.06	8.14
An2	205.64	20.29	3.71	2.22	2.59	10.16
An3	395.63	11.74	12.34	36.71	10.67	31.31
An4	418.20	7.48	4.47	33.11	10.16	31.83
An5	129.60	1.06	3.16	3.85	3.52	8.03
An6	106.51	0.97	2.43	2.61	3.40	8.00
An7	636.39	2.01	7.01	34.74	10.45	40.66
BAZ	241.19					
HAZ	562.03					
A1	42.62	5.85	4.02	2.40	5.09	1.18
A2	134.23	2.26	2.63	3.41	3.42	7.69
A3	198.75	1.65	1.78	31.48	2.71	2.11
A4	287.32	1.53	3.38	24.18	6.94	3.24
A5	27.93	1.35	6.49	1.71	1.74	5.49
A6	228.40	2.04	2.26	5.46	1.55	7.86
A7	382.24	3.06	4.42	19.70	7.93	2.51
BAZ	62.48					
HAZ	650.08					