MICROBIAL PRODUCTION OF ALIPHATIC HYDROCARBONS

Progress Report

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ABSTRACT

The neutral lipids of nine species of methanogenic bacteria, two thermoacidophiles, two alkalinophiles and 20 algal samples were analyzed. The major components were C_{30}, C_{25}, and/or C_{20} acyclic isoprenoid hydrocarbons with a continuous range of hydroisoprenoid homologues. The range or acyclic isoprenoids detected were from C_{14} to C_{30}. The neutral lipid composition from these bacteria, many of which exist in environmental conditions like those described for various evolutionary stages of the archaen ecology, resemble the isoprenoid distribution isolated from ancient sediments and petroleum. Therefore, these findings may have major implications to biological and biogeochemical evolution. In this connection, samples and cores from ancient sediments and future fossil fuel source beds are being analysed for these neutral lipids as well as the more polar isopranyl glycerol-ether lipids. The derivation of fossil fuels and the biomass accumulations are the focal points of this phase of the study.

Ancient and recent sediments, future source beds and local esturaries, are being enriched for microorganisms to establish a range and capability profile for hydrocarbon production. Diverse mixtures of algae and bacteria have been isolated. Only a relatively small percent of the microorganisms isolated demonstrated the ability to synthesize hydrocarbons; however, one particular algal isolate demonstrated, in preliminary studies, that it can synthesize hydrocarbons while in a green physiological stage that can account for more that 10% of its cell weight. Greater production is expected in the brown phase of growth.

Hydrocarbon biosynthesis studies were conducted in an attempt to better understand the conditions required to maximize hydrocarbon production. The program involved physical and chemical parameters as well as assays of specifically labelled precusors (Ketones, secondary alcohols, aldehydes, different glycerol ethers and peroxides) with a cell free enzyme system to measure their conversions to hydrocarbons. The results have indicated a complex one enzyme system is involved in condensation and reduction of two fatty acids into hydrocarbons.

Purpose

The proposed research was directed towards establishing a basis for the development of a microbial system for the commercial production of oily hydrocarbons. The proposed study consists of three parts: that dealing with screening of microorganisms for those that have the highest hydrocarbon producing potential; that dealing with physical-chemical factors on efficiency of hydrocarbon production; and the genetic basis of hydrocarbon production.

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The efforts of our first two years of our proposed four year program have been concentrated on screening of microbial systems for hydrocarbon producers and on the efficiency of hydrocarbon production. This report will, therefore, concentrate on these two parts.

A. Screening of microorganisms for hydrocarbon production.

A portion of the results obtained from the analyses of neutral lipid contents of microorganisms has been published (reprints enclosed), and the detailed data will not be repeated here.

The significance of the data reported in the five (5) publications is visualized in the similarities of the isoprenoid patterns of the Archaebacteria (Methanogens, Halophile and thermoacidophiles) to those found in ancient sediments and fossil fuels. These data provide evidence that many and perhaps all of the isoprenoid markers in sediments were synthesized by microorganism and that these markers were probably not derived via geological maturation.

The following data are being compiled in manuscript form for consideration for publications. The emphasis is on the detailed struc-

tural characterization of the neutral lipid components and the comparison of the structures to those isolated from sediments. The methyl branched hydrocarbons extracted from the microorganisms are listed in Table 1. The genealogical and biochemical significance of some of these findings has been reported in the enclosed reprints. In the following lines we will discuss the mass spectral evidence showing that the isoprenoids in sediments and petroleum could have been directly synthesized by methanogens, thermoacidophiles, halobacteria as well as other related microorganisms.

The mass spectrum of the $C_{15}H_{32}$ isoprenoid hydrocarbon, isolated from Thermoplasma, is shown in Fig. 1. The major fragments indicate a regular head to tail structure. The mass spectrum is identical to a C_{15}-isoprenoid isolated from the Windy Knoll bitumen (Derbyshire, England) and later identified as 2,6,10-trimethyldodecane. A regular head to tail structure is also assigned to the $C_{16}H_{34}$ isoprenoid (Fig. 2). A C_{16} isoprenoid monoene, isolated from Sulfolobus, was found to have also the regular head to tail skeleton. In addition, a C_{16}-iso alkane was isolated from Thermoplasma (Fig. 3b). The mass spectrum of this compound is compared with a C_{16}-iso alkane standard (2-methylpentadecane) (Fig. 3a.).

The mass spectrum of a C_{17}-isoprenoid isolated from a Sulfolobus sp. is shown in Fig. 4. There are indications that this isoprenoid has a regular head to tail structure corresponding to a 2,6,10-trimethyltetradecane.

The mass spectrum recorded in Fig. 4 is very similar to those reported for C_{17}-isoprenoids isolated from petroleum and sediments. A

C_{17}-isoalkane was identified in the neutral lipids content of Thermoplasma (Fig. 3d). Its mass spectrum is compared to a standard C_{17}-iso alkane (Fig. 3c).

The GLC retention values and the mass spectra of the C_{18}-isoprenoids isolated from Sulfolobus and Thermoplasma were identical. A C_{18} compound obtained from *M. vannielii* however, had different mass spectral characteristics, the results of which are not fully understood. The fragmentation pattern of the standard norpristane and of the C_{18}-isoprenoids from Thermoplasma and Sulfolobus are shown in Figs. 5 (a-c). The doublet at m/e 98 and 99, indicating the presence of a long straight chain and the major fragments at m/e 113, 169 and 183, are consistent with the structure of 2,6,10-trimethylpentadecane. The fragmentation patterns presented in Fig. 5a-c are very similar to those reported in the literature for C_{18}-isoprenoids isolated from shales and oils.

Pristane (2,6,10,14-tetramethylpentadecane) was detected in the neutral lipid content of Thermoplasma, Sulfolobus, Sulfolobus sp., *M. vannielii* and M. strain PS. The identification of the C_{19}-isoprenoids was done by comparison of GC retention and mass spectral data with those of authentic pristane. In addition, mass spectral evidence for a C_{19}-iso-alkane from Thermoplasma was obtained (Fig. 3e). Phytane ($C_{20}H_{42}$) was detected in the extracts of M. strain M.O.H. and Thermoplasma. A variety of other unsaturated (or cyclic) C_{20}-isoprenoids were also isolated.

A C_{21}-isoprenoid was isolated from Sulfolobus, and its mass spectrum is given in Fig. 6. The mass spectra of the isoprenoids is similar to that identified in Precambrians sediments, except that the head to tail structure showed a fragment at m/e 253, which cannot be formed from

the tail to tail structure (2,6,10, 15-tetramethylheptadecane). On the basis of the m/e 253 peak, which is clearly present in the mass spectrum of the C_{21}-isoprenoid from Sulfolobus, we suggest the structure 2,6,10,24-tetramethylheptadecane. This same structure, was assigned to the C_{21}-isoprenoid isolated from Precambrian sediments.

The mass spectrum of the C_{24}-isoprenoid isolated from Sulfolobus is presented in Fig. 7. Its fragmentation pattern is practically identical to those reported in the literature for a regular C_{24}-isoprenoid.

The mass spectra of the C_{25}-isoprenoid of *M. thermoautotrophicum* and *M. barkeri* are practically identical and are shown in Fig. 8b and Fig. 8c. The major fragments of the Sulfolobus isoprenoid seems to be a regular head to head structure.

The structure of 2,6,10,15,19-pentamethylcosane was assigned to the fragmentation pattern of the C_{25}-isoprenoids in Figs. 8b and 8c. The monounsaturated C_{25}-isoprenoid isolated from *M. barkeri* consists of a 2,6,10,15,19-pentamethylcosane skeleton.

The mass spectrum of the C_{26}-isoprenoid, isolated from Sulfolobus, is presented in Fig. 9. On the basis of the intense m/e peaks, a regular head to tail structure was assigned. The C_{28}-isoprenoid (Fig. 10) is also believed to have the regular head to tail skeleton. For the saturated C_{29}-isoprenoid from Sulfolobus, there is only chromatographic evidence; however, a mass spectrum of the corresponding C_{29}-monoene was obtained. It showed intense peaks at m/e 56, 126, 196, 266, 336 and 406 which are an indication of a regular head to tail structure.

From the large number of mass spectra obtained for various C_{30}-isoprenoids, we selected a few for discussion. In Fig. 11a is the mass spectrum of the C_{30}-isoprenoid from Sulfolobus. The fragmentation

supports a regular head to tail structure. On the basis of arguments developed earlier for the regular C_{25}-isoprenoid (Fig. 8a), a 2,6,10, 14,18,23-hexamethyltetracosane skeleton can be ruled out since a peak at m/e 379 (M-43) is not observed. Thus, a regular head to tail structure seems likely for the C_{30}-isoprenoid. The mass spectrum of the C_{30}-isoprenoid monoene, isolated from the same source, shows a strong molecular ion peak at m/e 420 and major fragments at m/e 126, 140, 196, 210, 252, 266, 280 and 336 suggesting also a 2,6,10,14,18,23-hexamethyltetracosane skeleton.

The mass spectrum of squalene (2,6,10,15,19,23-hexamethyltetracosane) is shown in Fig. 11b. The compound exhibits the expected fragmentation pattern. The mass spectrum of the corresponding C_{30}-isoprenoid monoene isolated from *M. ruminantium M-1* reveals a 2,6,10,15,19,23-hexamethyltetracosane.

There was solid evidence that the phytol side chain of chlorophyll is a biological precursor to phytane, one of the abundant isoprenoids found in sediments and petroleum. The formation of the lower isoprenoids from phytol was believed to occur through a series of oxidation, reduction or cracking mechanisms, which was assumed to be prevalent in the geological environment. Although it is known from diagenesis experiments that microbial activity or high subsurface temperatures can alter the chemical integrity of compounds such as phytol, there is no experimental evidence which assesses the role of these factors on the distribution of isoprenoids in sediments and petroleum. Our present study shows that the majority of these isoprenoids are present as neutral lipids or intermediate metabolites in the microorganisms.

In Table 2, the cellular lipids are compared to a series of isoprenoids isolated from geological samples up to 2.7×10^9 years old. The isoprenoids of both groups are in the same carbon range. All compounds listed (Table 2) have a regular head to tail structure except for squalane or squalene which have resulted from a tail to tail condensation of two farnesyl residues.

Squalene and hydrosqualenes are the major isoprenoid in most methanogenic bacteria (Table 1). The fully hydrogenated form, squalane, has been identified in Nigerian petroleum. Squalane is one of the few isoprenoids in sediments or petroleum known to have a tail to tail skeleton like those predominantly found the methanogens. A C_{30}-isoprenoid structure with a regular head to tail skeleton was reported in Bell Creek crude oil. Compounds structurally similar to this are synthesized by Sulfolobus. The identified isoprenoids from Sulfolobus in the range from C_{16} to C_{30} are of the regular head to tail skeleton; there was no evidences for the formation of the irregular tail to tail structure. Of further interest is the isolation of a C_{17}-isoprenoid from Sulfolobus sp. as well as from ancient sediments. This hydrocarbon is less likely to be generated from phytane in a diagenetic pathway because it would involve the cleavage of two carbon-carbon bonds.

As minor components, several iso-alkanes have been isolated from Thermoplasma (iso-C_{16},-C_{17},-C_{19}). These compounds which are also known to occur in algae have been detected in various sediments. Besides the branched hydrocarbons, which are the major constitutents of the examined microorganisms, several other groups of substances were detected. Various samples contained a sequence of methyl branched fatty acids; cyclic terpenoids, were found in two samples, as well as an entire series of methyl branched alkylbenzenes.

In conclusion, this phase of our study shows that the majority of isoprenoids detected in sediments and petroleum are also present as cellular products or intermediate metabolites in methanogens and in specific thermoacidophiles. The fact that these microorganisms may have dominated the biosphere in the early history of our planet suggests that many isoprenoids isolated from ancient geological samples may actually have been synthesized by microorganisms as those described here. It also suggests that current anaerobic environments, such as the geopressurized natural gas zones of the Gulf of Mexico, and other special environments, are the abode of these unique and interesting microorganisms currently called Archaebacteria.

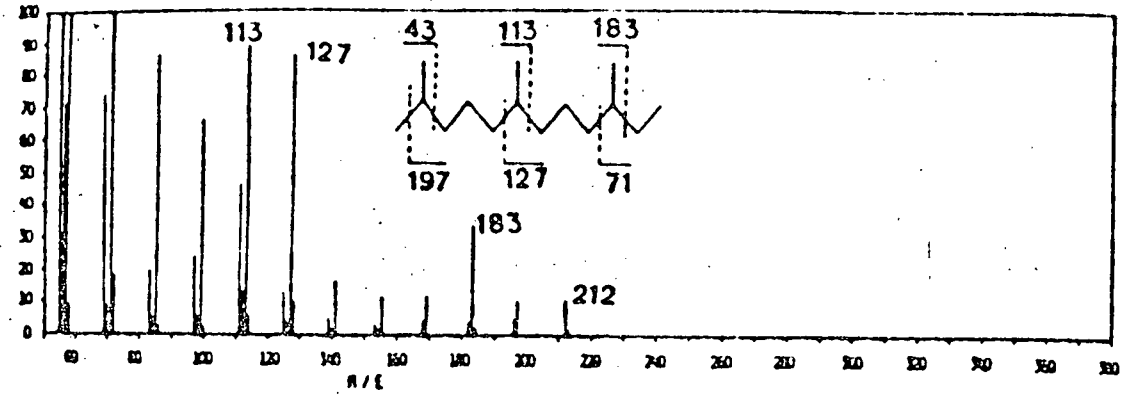


Fig. 1: Mass spectrum of a C_{15}-isoprenoid isolated from Thermoplasma.

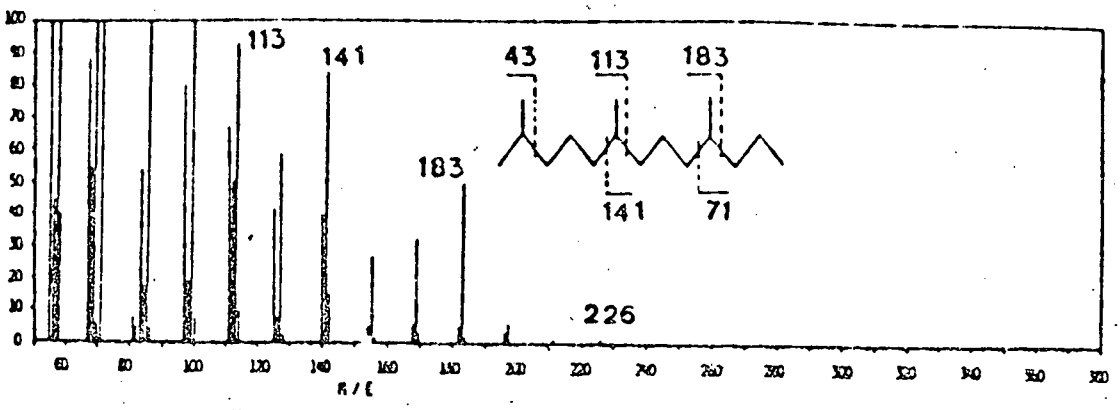
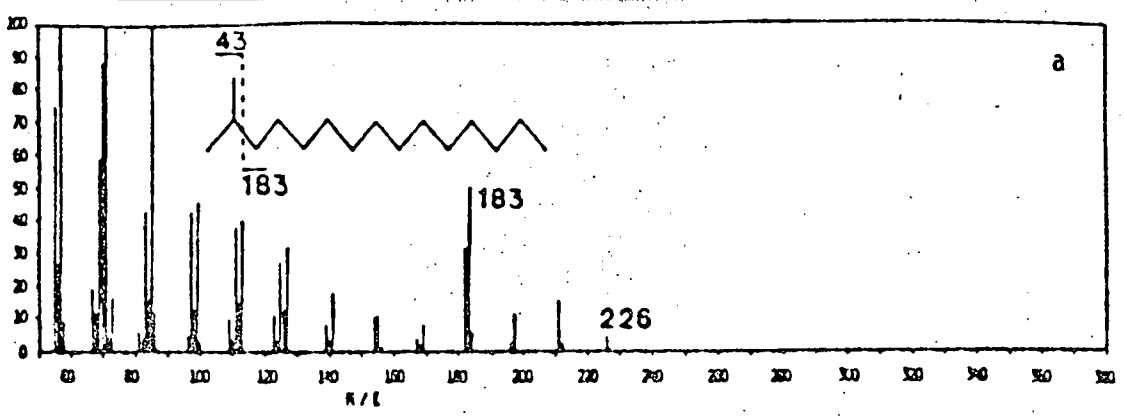


Fig. 2: Mass spectrum of a C_{16}-isoprenoid isolated from Thermoplasma.

Fig. 3a: Mass spectrum of a standard C_{16} iso-alkane (2-methylpentadecane).
Fig. 3b: Mass spectrum of a C_{16}-iso-alkane isolated from Thermoplasma.
Fig. 3c: Mass spectrum of a C_{17}- iso-alkane (2-methylhexadecane).
Fig. 3d: Mass spectrum of a C_{17}-iso-alkane isolated from Thermoplasma.
Fig. 3e: Mass spectrum of a C_{19}-iso-alkane isolated from Thermoplasma.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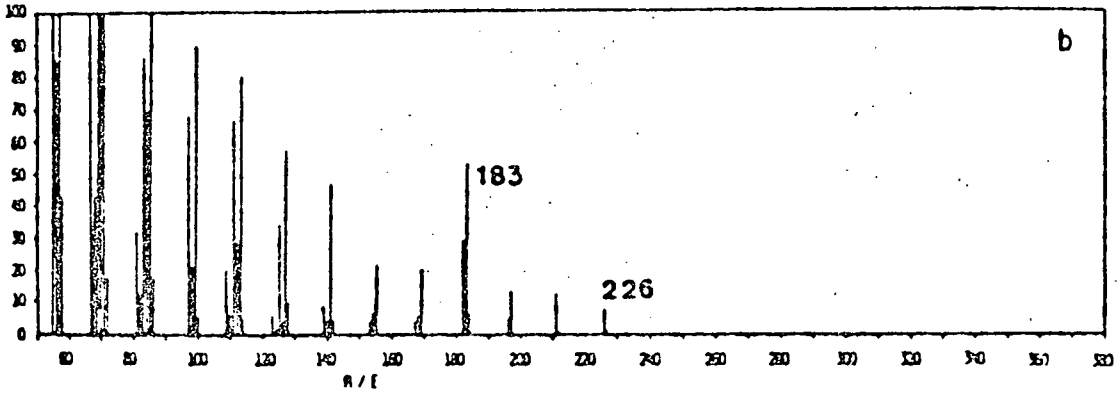
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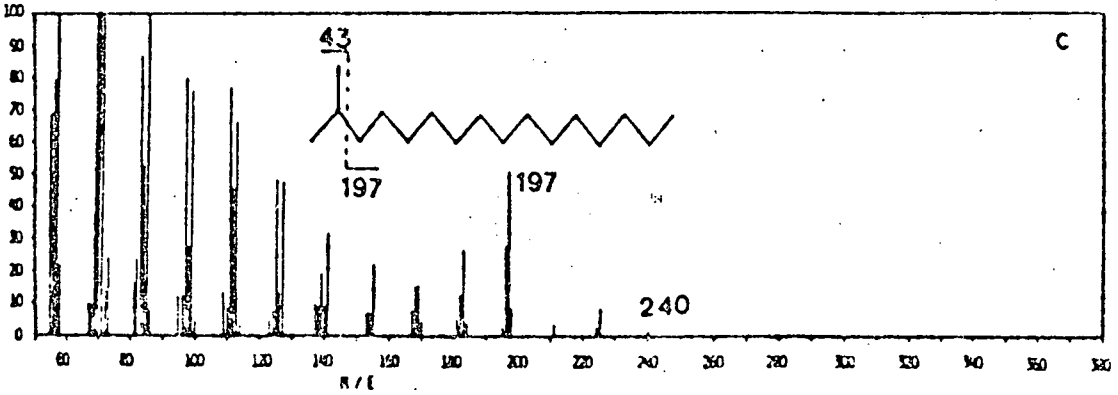
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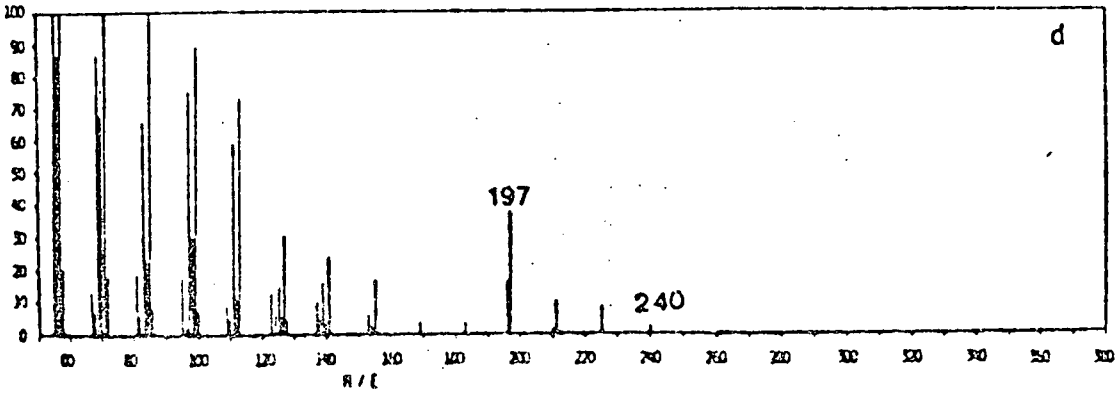
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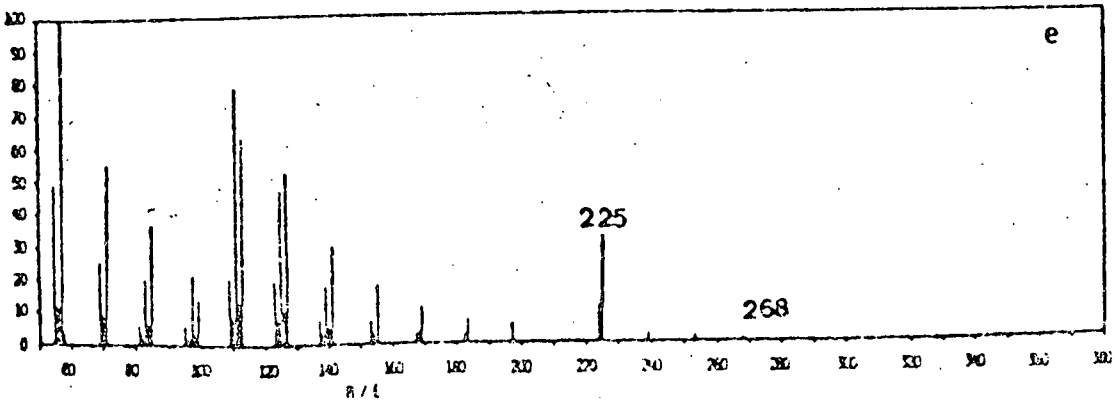
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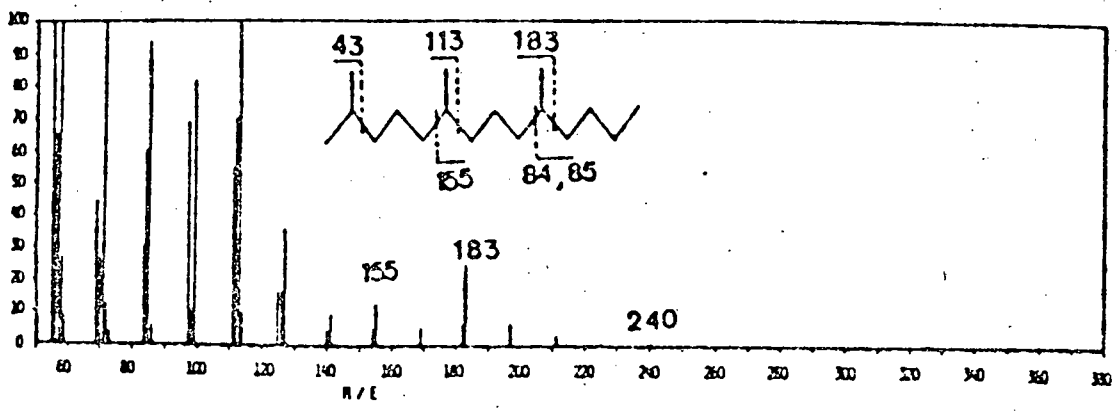


Fig. 4: Mass spectrum of a C_{17}-isoprenoid isolated from Ferrobolus.

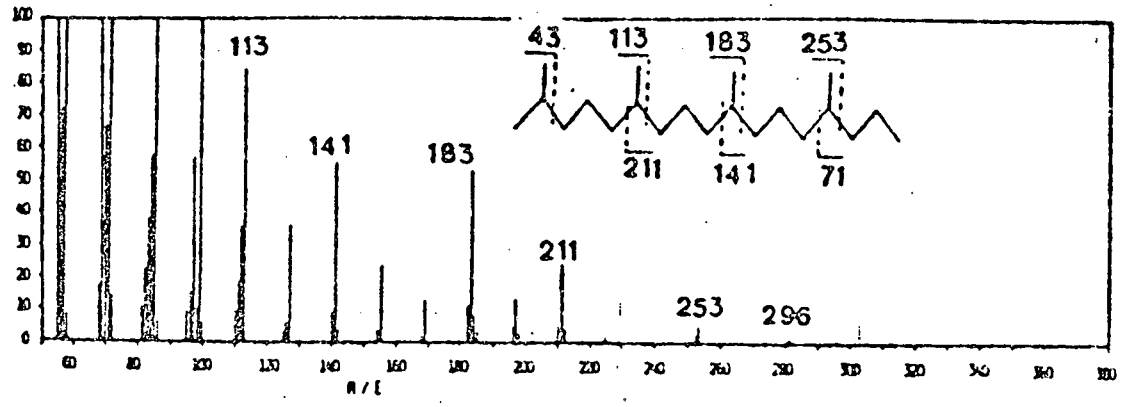


Fig. 6: Mass spectrum of a C_{21}-isoprenoid isolated from Sulfolobus.

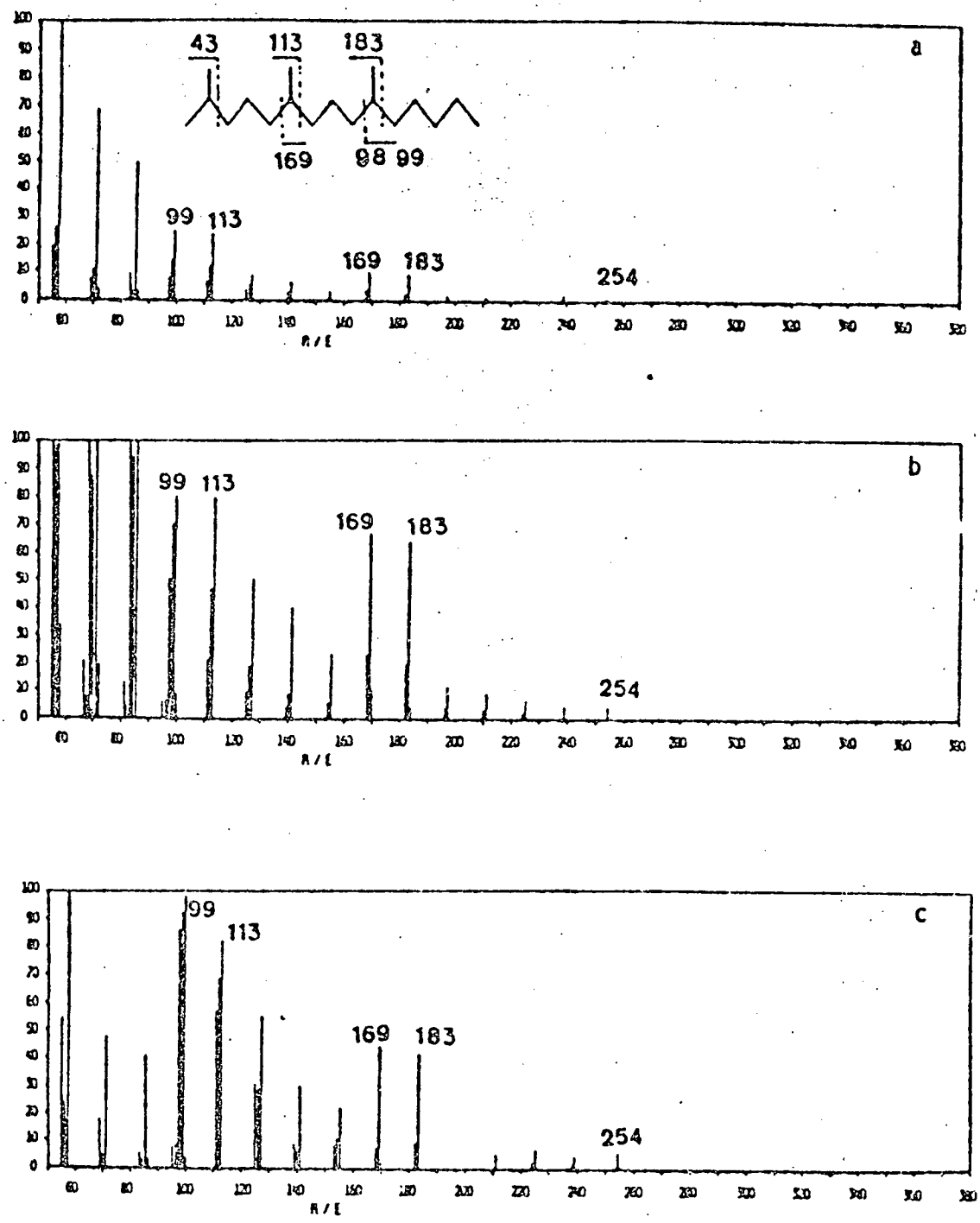


Fig. 5a: Mass spectrum of a standard C_{18}-isoprenoid (nonpristane).
Fig. 5b: Mass spectrum of a C_{18}-isoprenoid isolated from Thermoplasma.
Fig. 5c: Mass spectrum of a C_{18}-isoprenoid isolated from Sulfolobus.

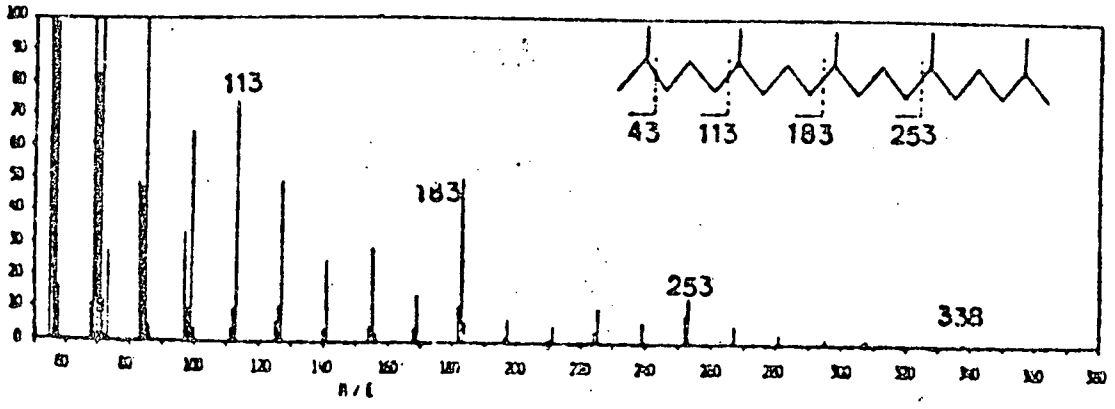
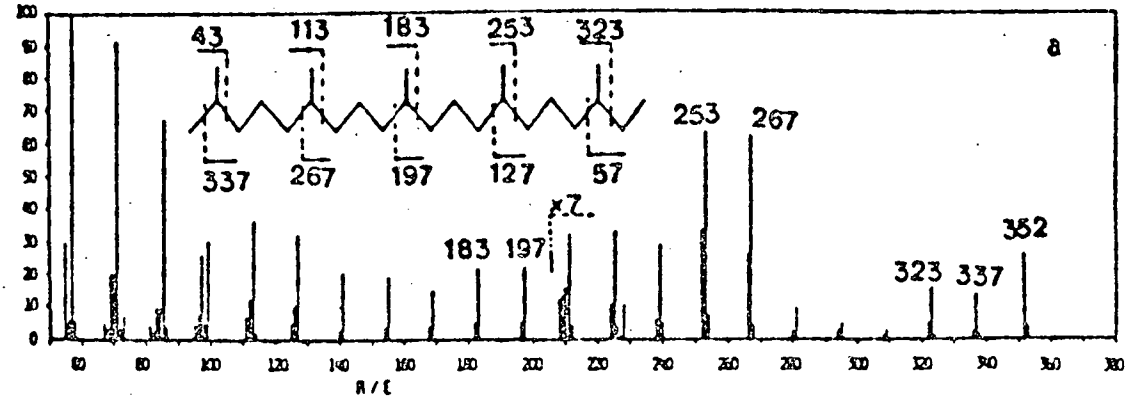
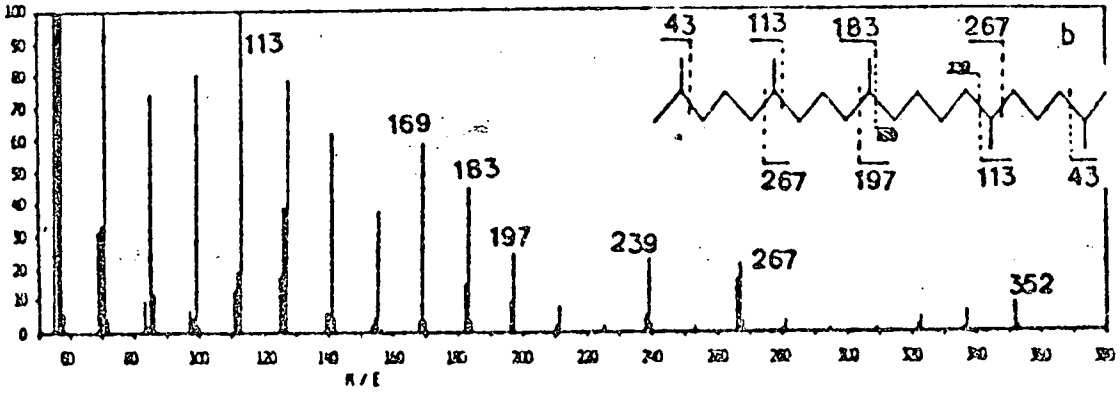


Fig. 7: Mass spectrum of a C_{24}-isoprenoid isolated from Sulfolobus.





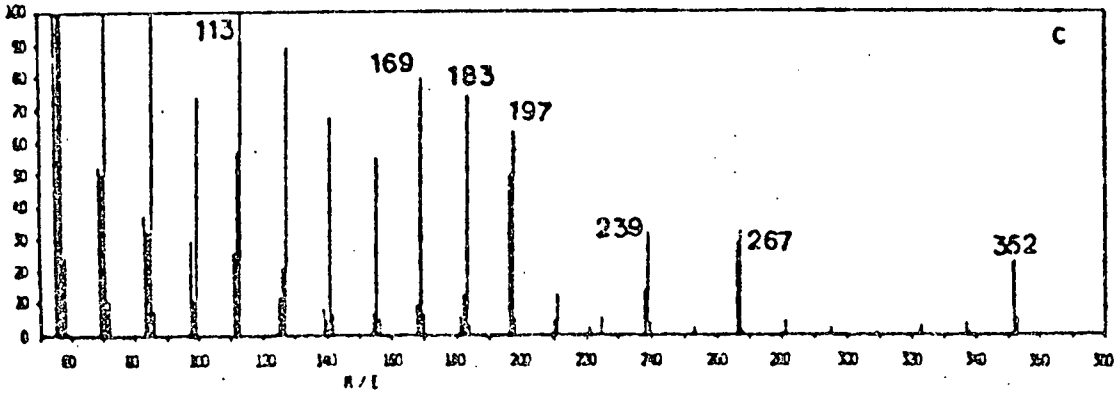


Fig. 8a: Mass spectrum of a C_{25}-isoprenoid isolated from Sulfolobus.
Fig. 8b: Mass spectrum of a C_{25}-isoprenoid isolated from *Thermoautotrophicum*.
Fig. 8c: Mass spectrum of a C_{25}-isoprenoid isolated from *M. barkeri*.

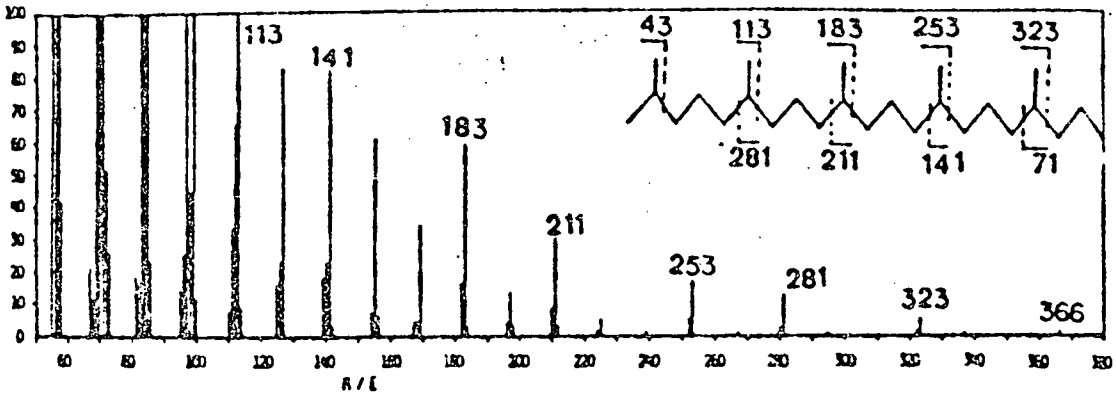


Fig. 9: Mass spectrum of a C_{26}-isoprenoid isolated from Sulfolobus.

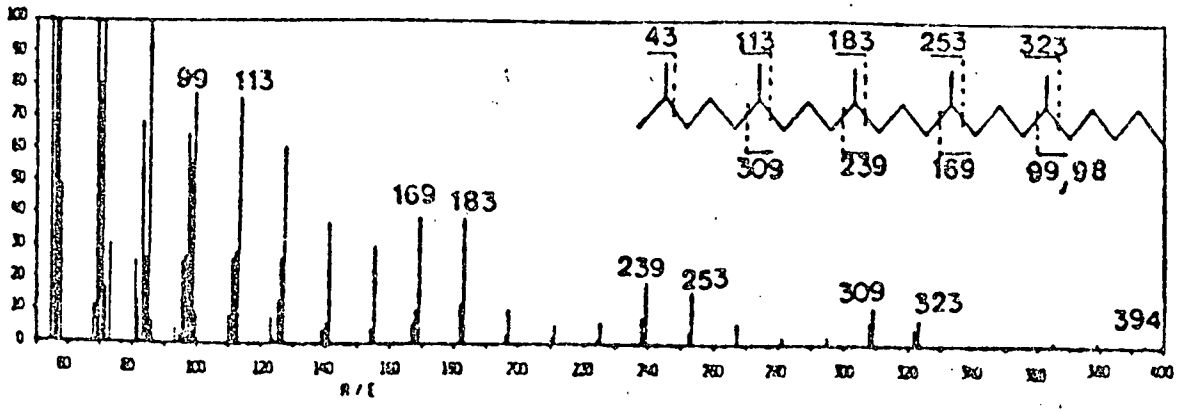
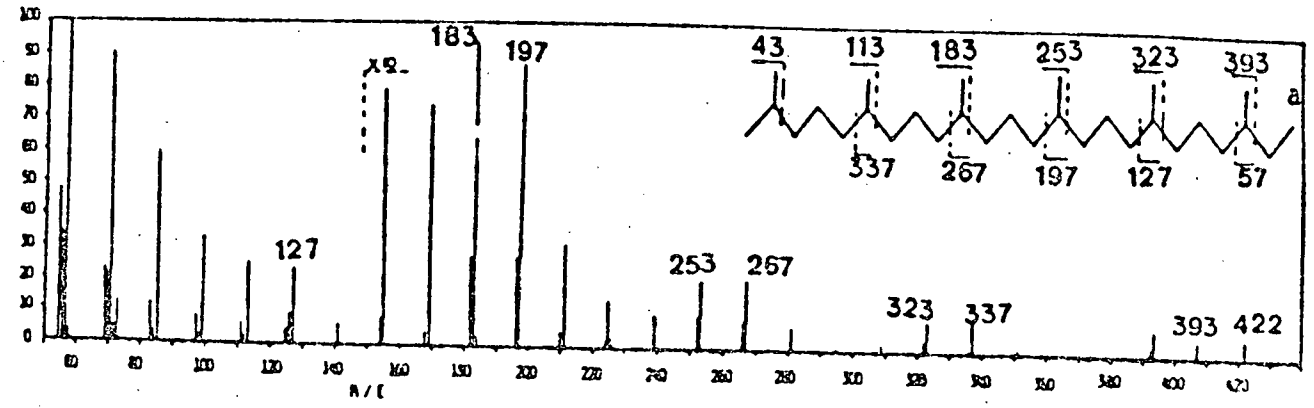


Fig 10: Mass spectrum of a C_{28}-isoprenoid isolated from Sulfolobus.



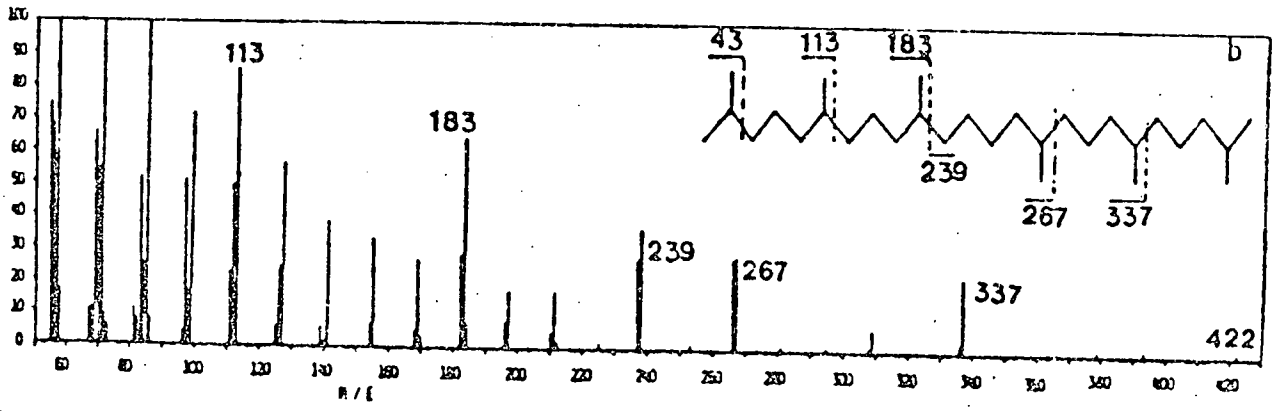


Fig. 11a: Mass spectrum of a C_{30}-isoprenoid isolated from Sulfolobus.
Fig. 11b: Mass spectrum of a C_{30}-isoprenoid (squalane).

Molecular ion	M. strain AZ (a)	M ruminantium PS	M. ruminantium M-1	M. thermoautotrophicum	M. strain M.o.H.	M. vanniellii	M. strain PS	M. hungatii	M. barkeri	Thermoplasma acidophilum (b)	Sulfolobus acidocaldarius (b)	Ferrolobus (b)
$C_{15}H_{32}$										3.8		
$C_{16}H_{34}$										5.4		
$C_{16}H_{32}$											<0.1	
$C_{16}H_{30}$										1.5		
$C_{17}H_{36}$										<0.1		1.0
$C_{17}H_{34}$										<0.1		
$C_{18}H_{38}$						0.8				2.4	<0.1	1.6
$C_{18}H_{36}$						1.0						
$C_{18}H_{34}$						1.2						
$C_{19}H_{40}$					<0.1	0.7	0.5			2.6	0.5	6.4
$C_{19}H_{38}$											0.3	
$C_{20}H_{42}$	<0.1				5.8					0.8	1.2	15.1
$C_{20}H_{40}$	<0.1				4.0					1.9	<0.1	
$C_{20}H_{38}$					2.8	0.3					<0.1	0.5
$C_{20}H_{36}$					1.5	1.0					<0.1	
$C_{20}H_{34}$	<0.1				1.0	1.3				3.1		
$C_{21}H_{44}$												5.0
$C_{24}H_{50}$											2.1	
$C_{24}H_{48}$											0.5	
$C_{25}H_{52}$									39.5		6.8	
$C_{25}H_{50}$				15.0					26.3		3.4	
$C_{25}H_{48}$				<0.1					13.5			
$C_{25}H_{46}$		0.2				0.8	0.8		13.0			
$C_{25}H_{44}$						7.0						
$C_{25}H_{42}$		2.0				16.5				7.2		
$C_{26}H_{54}$											5.1	
$C_{28}H_{58}$											2.7	
$C_{28}H_{56}$											1.9	
$C_{29}H_{58}$											7.1	
$C_{30}H_{62}$				<0.1							8.6	
$C_{30}H_{60}$			3.0	<0.1							1.3	
$C_{30}H_{58}$			6.2	2.5							0.4	
$C_{30}H_{56}$		0.6	9.3	7.5			18.1					
$C_{30}H_{54}$		3.0	16.0	20.0	3.7	0.3	52.0	1.8				
$C_{30}H_{52}$		12.0	22.5	27.4	10.6	4.1	13.2	10.0				
$C_{30}H_{50}$	64.0	82.0	41.5	24.9	69.0	64.0	2.5	88.0		34.0		

TABLE I

TABLE 2

C_{15} - C_{30} ISOPRENOIDS FOUND IN ANCIENT OIL SHALES AND PETROLEUM, AND THEIR OCCURRENCE IN METHANOGENS, AND THERMOACIDOPHILES.

Squalane has been identified only in *M. thermoautotrophicum*, however, most methanogenic bacteria contains squalene and various squalene analogs only in degree of hydrogenation.

ISOPRENOID	GEOLOGICAL SAMPLE	ISOPRENOID	MICROORGANISM
C_{15} - C_{20}	Green River Shale (40)	C_{15} - C_{20}	Thermoplasma
C_{15} - C_{21}	Nonesuch Shale (5, 36, 41)	C_{16}, C_{18} - C_{20}	Sulfolobus
C_{16}, C_{18} - C_{21}	Antrim Shale (5,36)	C_{17} - C_{21}	Ferrobolus
C_{18} - C_{21}	Soudan Shale	C_{18}, C_{19}	M. Vannielii
		C_{19}, C_{19}	M. M.o.H.
		C_{20}	M. PS, M. AZ
C_{21} - C_{25}	African Cretaceaus Shale	C_{21}	Ferrolobus
C_{30} (Squalane)	(10)	C_{24}, C_{25}	Sulfolobus
		C_{30} (Squalene)	Methanogens
C_{22} - C_{25}	Bell Creek Crude Oil (8)	C_{24}, C_{25}	Sulfolobus
C_{24}, C_{25}, C_{28} C_{30}	Costa Rican Seep Oil (12)	C_{24}, C_{25}, C_{28} C_{30}	Sulfolobus
C_{30} (Squalene)	Nigerian Petroleum (38)	C_{30} (Squalene)	Methanogens

Studies also in progress are attempting to determine the biomass of sediments, through an analytical procedure, developed in our Laboratory, for detecting isoprenyl ether lipids of cores from peat bogs of Great Lakes, Green river shale, coal, algal mat sediments of Baffin Bay Texas, Great Salt Lake sediments, Monterey shale, Texas Rock (15 cores from 3 counties in central Texas) and a wide variety of sediments collected around the world by Dr. R. Wolfe. Although the studies are only in the initial stages, the preliminary findings are sufficient to predict that data will be obtained to substantiate the degrees of involvements of microorganisms in sediment formations. These findings may well influence and change the present thoughts on geologic markers and biogeology. These studies are vital to a better understanding of the endogenous microflora that existed in petroleum deposits and those that may be involved in the formation of future source beds.

Enrichment of many of these same sediments for recovery of the endogenous population is important to establish a range and capability profile for hydrocarbon production. Our approach to screening microorganisms has centered on those populations contained within sediments judged as future fossil fuel source beds.

Arrangements have been made with Dr. J.H. Weber of DOE, Laramie Wyoming for specific samples from different locations under the control of the Laramie Energy Technology Center. Although they have not been received, it is anticipated that they will be available in the near future. These are critical samples in our program in view of our previous findings from studies on oil shale. This was reported to DOE in our Progress Report for period August 1977 to July 1978.

The following samples have been obtained from Shell Development Company, Belaire, Texas Research Center after considerable communications and negotiations:

Baffin Bay, Texas, Algal Mat Sediments (Recent)

1) 78TXKK-3-400. 40-80cm depth section of our core #3. Mostly mud and poorly preserved algal mats.
2) 78TXKK-6. Composite sample of surface (top 1.0cm) algal mat just above mud interval deposited during hurricane Beulah in 1967.
3) 78TXKK-9. First algal mat below Beulah mud. 3-6cm depth.

Great Salt Lake Sediments, North Arm (Recent)

4) Rosel Point mud, surface sediments in shallow water (<1' water depth). Possible contamination with oil from nearby seepage.
5) Little Valley Harbor bottom mud, about 8' water depth.

California Rock Sample

6) CAL-S-609. Monterey Shale (Miocene). Core sample from Shell Security Fee #39 at 5750-5756'8" depth, Santa Barbara County. A marine source rock at a very early stage of organic diagenesis (Vitrinite Reflectance 0.44).

Texas Rock (Coal) Sample

7) HA-S-314D. Yegua Formation (Eocene) outcrop sample, fayette County. At a very early stage of organic diagenesis (Vitrinite Reflectance 0.31).

These samples were received in late summer and work on enrichments for microorganisms have barely begun; therefore, no definitive data has been obtained.

We are also negotiating with an Illinois Geological Survey group which is conducting an evaluation of methanogens in bogs and other sediments. They have agreed to send us representative samples to survey the microbial population with the exception of the methanogens. Hopefully, these samples will be in our laboratory in the next couple of weeks.

Since much of our attempts to obtain representative source bed samples have been greatly delayed, we chose an area proposed by Colorado Geological Survey to be rich in organic deposits as a site for microbial enrichments. The area of Colorado was sampled two times a month for four months by undergraduate students under the auspices of special studies in microbiology under the direction of Dr. William Boyd of our Department of Microbiology. From a randomized sampling, five feet core samples were taken and enriched for microorganisms.

The methodologies employed for all samples obtained for microbial enrichment studies included procedures for obtaining thermophiles, mesophiles, psychrophiles, acidophiles and alkalinophiles that are either autotrophs or heterotrophs. This program also included the enrichments for algae and cyanobacteria. From this program, 14 thermophiles, 62 mesophiles, 5 psychrophiles, 3 algae and 9 cyanobacteria were isolated. Cell preparations of each isolate were extracted for total lipids, which were fractionated by column chromatography on silicic acid. Both the hexane eluates (acyclic non-isoprenoid hydrocarbons) and benzene eluates (cyclic and acyclic isoprenoid hydrocarbons, esters, acids) were saved for gas chromatographic analyses. No components were detected in the hexane fractions of the Thermophiles; the benzene eluates have not yet been examined. From the collected 62 mesophiles, only the hexanes from 40 of them have been analyzed. Of these 40 cultures, 6 of them had aliphatic hydrocarbon contents. This was quite significant in that the contents were higher than 1% of dry cell weight (exact amounts are being determined) and that the cultures were Gram-positive or Gram-negative, rods, cocci or branching filaments. Each of the hydrocarbon profiles were distinctly different from the other. In the case of isolate M-16,

the range of components was from C_{20} to C_{36} with major component of C_{29}, C_{30}, C_{31} and C_{34}. Rough estimates put the content at >3% of cell dry weight. For isolate M-22 the hydrocarbons consisted of C_{30}, C_{32} and C_{34}; for isolate M-25 the contents were C_{25}, C_{28}, C_{29} and C_{30}; for isolate M-26 it was a family of branched isomers of C_{29}; for isolate M-27 the hydrocarbon component was only a C_{29}; for isolate M-27 the hydrocarbon component was only a C_{29}; and for M-32 contents consist of C_{28} and C_{29}. The exact structural details have not yet been determined. None of the benzene fractions have yet been characterized.

The cultivation of the psychrophiles and the extractions and fractionations have been performed but the samples have not yet been analyzed.

Approximately 15 algal samples enriched from the Mirror Lake region are currently being cultivated.

Since the initiation of this contract period in February 1979, we had also looked at Poudre River algae and diatoms. None of these 20 samples contained appreciable quantities of straight chain aliphatic hydrocarbons. However, the isoprenoid contents were readily measurable. The C_{20}, C_{25}, and C_{30} isoprenoids and hydroisoprenoids were identified. These components related to the types found in the archaebacteria (methanogens, thermoacidophile, and halophiles). These algal samples, however, did not contain the glycerol-ether derivative that are a characteristic chemical markers of the archaebacteria.

Alkalinophiles were examined to determine if these bacteria were generically similar to the archaebacteria. These organism growing optimally at pH's above 10 are classified as bacteria from an extreme or unusual environments, a property shared by most archaebacteria. Lipid analyses

showed the isoprenoid markers similar to that found in the archaebacteria, and which are apparently common to most microorganisms but they did not contain measurable amounts of aliphatic hydrocarbons and no glycerol-ether lipids.

Attempts to secure a variety of strains of *Botryococcus braunii* have been unsuccessful. There have been no responses to our letters and phone calls to laboratories supposedly classifying these algae. We are still seeking information, through inquiries, on those investigators that have Botryococcus strains. We have been frustrated by the absence of this alga in local estuaries. Botryococcus blooms usually frequent the ponds and wells of northern Colorado; however, we are continuing the search for this alga in local ponds and wells. Perhaps these blooms may appear more prevalent this fall. A problem we have encountered, however, is the poisoning of lakes, ponds and wells by farmers to eliminate algal blooms.

The most frequent isolate being collected from local estuaries are the blue green algae. The second most common are mixtures of green and golden brown algae. Twenty-five representative samples have been collected, most of them subcultured and analyzed. All but two isolates produced detectable quantities of aliphatic hydrocarbons. It was gratifying to find typical chromatographic profiles that delineated the identity of most algal samples. Four specific chromatographic patterns were observed for the blue green algae; these patterns were methylated 17:0 and 17:1; methyl branched 15:0; 20:6 (polyunsaturated); and, a mixture of methylated 17:0, 17:1; 15:0 and 15:1. All of these type patterns and their identities have been previously reported, however,

one potentially interesting fact was revealed. While the reported hydrocarbon content of these algae are typically in the range of 0.005 to 0.6% of cell dry weight, we found that the hydrocarbon content for all the blue green algae was 0.8 to 2.5%. We are tentatively attributing this increase in yield to our preparatory and handling procedure. We are taking the active green phase of the cells and cooling them down in a refrigerant unit accompanied by shortened photoperiods. In essence, we are attempting to bring the cells out of the green phase and into the brown or "resting" phase. Our rationale is simply taking from what has been observed for hydrocarbon yields for *B. braunii* which showed an increase of five times the hydrocarbon content by converting from green active phase to brown resting phase. This is an area we are going to pursue further with the coming of fall and winter. I suspect, if we can find the algae, that the brown phase will reveal the true hydrocarbon producing potential of all algae.

The algae samples other than the blue-greens were also analysed. The hydrocarbon profiles are in the range from C_{16} to C_{32}. Most of the hydrocarbon contents were negligible with the exception of sample Ag-5. This particular one provided us with hydrocarbons in the 10% dry cell weight range with a complex of components in the range of C_{20} - C_{30}. The major component cochromatographed with a branched C_{27} standard. The components have not yet been fully identified. We are now attempting to identify this alga for further studies.

The acyclic isoprenoid hydrocarbon content of all the present isolates are ready for analysis, however, only one GLC is available in our laboratory for analysis, and we must work into the entire laboratory

schedule. Over 100 hydrocarbon fractions are ready for analysis but on an average, only seven samples can be adequately surveyed in a 14 hour work period.

Progress has been somewhat slower than we expected but, nonetheless, encouraging at this early state of the screening with one of the samples isolated with high hydrocarbon content.

B. Effects of chemical-physical factors on hydrocarbon production.

The alteration of growth phases by 1) lowering temperature, 2) reducing growth rates through limiting growth factors, and 3) maintaining viable cells in mineral media that will support metabolism but not rapid cell growth and replication have provided for enhanced hydrocarbon production by algae and bacteria. The only explanation for these occurrences, at this stage of the investigations is that 1) a fluid membrane must be maintained and at reduced temperatures the biosynthesis of lipids in specific cells are switched to the formation of more hydrocarbon oils which preserve the fluid property and which maintain an eH potential (for more detailed explanation, see enclosed reprints, T. G. Tornabene, J. Mol. Evol. *11*:253-257, 1978); or 2) increased production of free fatty acids which induce enzymes for coupling of two acids into a less toxic hydrocarbon form. We feel that these types of studies, many of which are in progress, are very important to our understanding of the hydrocarbon producing potential of microorganisms.

The prevention of hydrocarbon biosynthesis through heavy metal poisoning resulted in ketone synthesis whose branching characters were equal to those of the hydrocarbons. Reduction of fatty acid biosynthesis resulted in neither hydrocarbon or ketone accumulations. In an attempt to better understand the hydrocarbon biosynthesis process, extensive

efforts have been conducted to define the specific precursor forms in the mechanism. In this study we have synthesized in our laboratory specifically radioactively labeled ketones, secondary alcohols, aldehydes, fatty acids, phytanyl glyceryl ether, hexadecyl-1-enyl ether, epoxides, and peroxides to test for their suitability to be incorporated into hydrocarbons. One sequence of experiments involving reductive extraction of precursors is still in progress. Hopefully, the experiments in progress will enable us to terminate this phase of the project by the end of the current contract period. At that time we will formulate in manuscript form the exact findings of our hydrocarbon synthesis studies and submit it to DOE.

C. Personnel.

1. T. G. Tornabene, principal investigator, three-fourths of time has been devoted to this project. Approximately two-thirds time will be devoted from September to January 31.
2. S. Wu-Hunter, post-doctoral person, full time.
3. P. S. Eastman, graduate student, one-half time.
4. R. Lloyd, graduate student, one-half time.
5. Others who have participated or collaborated on the project are:
   a. T. A. Langworthy, University of South Dakota, cultivation of thermoacidophiles and polar lipid characterization.
   b. R. Wolfe and W. Balch, University of Illinois, cultivation of methanogens.
   c. G. Holzer, University of Houston, mass spectral analyses and interpretation.

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