Studies on Lipids of Soil Micro-organisms with Particular Reference to Hydrocarbons

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SUMMARY

The lipids of soil micro-organisms harvested from simple and complex media varied from 2 to 20% in bacteria, 10 to 20% in fungi, 2.5 to 15% (w/w) in algae. The bulk of the lipid usually consisted of polar compounds; paraffinic hydrocarbons comprised 0.008 to 2.7% in bacteria, 0.04 to 0.7% in fungi, 0.08 to 2.9% (w/w) in algae. Lipid contents of algae were more affected by growth medium composition than were those of the bacteria and fungi. Gas-liquid chromatography showed that the hydrocarbons were paraffins in the range C_{16-36} . The hydrocarbon patterns varied with species and growth medium. A peak in the range C_{27-31} was usual in bacteria with sometimes a minor peak in the range C_{18-22} . The fungi exhibited slightly more stable hydrocarbon patterns (except *Trichoderma viride*) than bacteria and most showed major and minor paraffin peaks in similar regions.

The algae showed a peak at C_{17} regardless of the growth medium but *Tetraspora gelatinosa* showed an increase in C_{25} and C_{27} paraffin content when grown with acetate. The ratio of paraffin chains consisting of odd numbers of carbon atoms to those containing even numbers of carbon atoms was around unity for bacteria and fungi, also for *T. gelatinosa* when it was grown on CO_2 as sole carbon source. The *Nostoc* sp. and *T. gelatinosa* (grown with CO_2 plus acetate) contained predominantly C_{odd} paraffins.

INTRODUCTION

The first report of the isolation of hydrocarbons from microbes was by Jankowskii & ZoBell (1944). Oppenheimer (1965) reported the production of 'hydrocarbon-like' materials in *Desulfovibrio desulfuricans* and in mixed cultures of micro-organisms. Other investigations of microbial hydrocarbons have been reported by Albro & Huston, 1964; Oró, Tornabene, Nooner & Gelpi, 1967; Tornabene, Gelpi & Oró, 1967; Tornabene, Bennet & Oró, 1967; Tornabene & Oró, 1967; Han *et al.*, 1968.

The range of *n*-paraffins isolated from fungi is very similar to that in bacteria although, as with bacteria, the composition of the mixture varied with the species investigated. Merdinger & Devine (1965) and Merdinger & Frye (1966) demonstrated *n*-alkanes in the range C_{16-39} with a peak at C_{22} in *Debaryomyces hansenii*; glucose carbon was incorporated into these hydrocarbons. Oró, Laseter & Weber (1966) isolated a similar range of hydrocarbons from fungal spores and noted a C_{odd} predominance with peaks at C_{27} , C_{29} and C_{35} . Most investigations of algal paraffins

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indicate a carbon preference index (ratio of C_{odd} to C_{even} compounds) of unity. Patterson (1967), however, noted an increase in concentration of the C_{25} and C_{27} paraffins in *Chlorella vulgaris* when it was grown chemo-organotrophically. Other workers have noted a lower boiling point range (C_{15-25}) of *n*-paraffins in certain algae (Oró *et al.* 1967; Han *et al.* 1968), sometimes with peaks around C_{17} . The present study reports patterns of alkanes produced by soil micro-organisms to determine what role if any these alkanes played in the hydrocarbon spectrum of the soil.

METHODS

Bacteria. These were dominant species isolated from an upland moorland soil. Methods of isolation and details of the site have been reported previously (Jones & Edington, 1968). The species were *Arthrobacter* sp. NCIB 10407, *Micrococcus* sp. NCIB 10405, *Corynebacterium* sp. NCIB 10406, *Bacillus* sp. NCIB 10404, *Mycobacterium* sp. NCIB 10403, and a Gram-negative rod, NCIB 10408.

Fungi and algae were isolated from the same soil samples and so were *Penicillium* sp., *Aspergillus* sp., *Trichoderma viride*, *Saccharomyces* sp., *Tetraspora gelatinosa* and *Nostoc* sp.

Preparation of material. The basal succinate medium (BSM) for the fungi or bacteria contained (g./l. distilled H_2O): Na succinate, 10.0; K_2HPO_4 , 1.5; KH_2PO_4 , 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 2.0; KNO_3 , 2.0; NaCl, 0.1; $FeCl_3$, 0.01; yeast extract (oxoid), 1.0; pH 7.2 for bacteria, 5.0 for fungi. KNO_3 was omitted for *Mycobacterium* sp. The complex medium used was tryptone soya broth (oxoid) (TSB) adjusted to pH 5.0 for fungi. Slope cultures of bacteria and fungi were subcultured into 20 ml. starter cultures of BSM which were inoculated into 500 ml. BSM or TSB and subsequently into 9 l. batches of the respective media. The micro-organisms were harvested when they entered the stationary phase. The basal medium for *Tetraspora gelatinosa* was Knop solution (g./l. dist. H_2O): KNO_3 , 1.0; $Ca(NO_3)_2$, 0.1; K_2HPO_4 , 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; $FeCl_3$, 0.001, pH 7.0 (code 1 in Tables). The *Nostoc* sp. was grown on the medium (code 3 in Tables) of van Baalen (1965), pH 7.0, which also contained 6 ml. of van Baalen's 'H₅' trace element solution. Na_2CO_3 (0.020 g./l.) was added to both media. The algae were also grown with 10^{-2} M-Na acetate (Hoare, Hoare & Moore, 1967) added (code 2 and 4 in Tables).

The cultures were grown under conditions of forced aeration, bacteria and fungi at 25° in the dark and algae at c. 20°, 6 in. from 3-ft. fluorescent lamps (c. 800 ft. candles). Bacteria were harvested in the 6 l. swing-out head of the Mistral 6 L centrifuge (MSE Ltd.) at 1570 g for 30 min., the filamentous fungi were harvested by filtration, and the algae by using the continuous head attachment on the MSE 17 centrifuge at 3000 rev./min. They were washed twice and dried overnight under vacuum over P_2O_5 . The dried material was ground in a pestle and mortar.

Extraction procedures. The ground samples in fat-free thimbles were extracted with a 2+1 (v/v) chloroform+methanol mixture for 8 hr in a Soxhlet-type extractor (ASTM D297, Gallenkamp & Co. Ltd.). Solvent was removed *in vacuo* at 40° to give the 'total lipid fraction'.

Column chromatography. The methods used were based on those of Evans, Kenny, Meinschein & Bray (1957), Meinschein & Kenny (1957), Kolattukudy (1966), Oró *et al.* (1967). The total lipid fraction on a silica gel column (prewet with *n*-hexane) was

successively eluted with *n*-hexane, benzene and methanol to give paraffinic, aromatic and polar lipid fractions respectively. The adsorbents were washed with chloroform + methanol (2 + 1), before activation at 110° overnight for silica gel (100 to 200 mesh) and 48 hr at 165° for alumina. If it was not used immediately the adsorbent was stored in the vacuum oven over P₂O₅ at 50°.

The adsorbent:total lipid ratio was never less than 300:1 (w/w) and the adsorbent:paraffin ratio never less than 1000:1. The paraffins were recovered from the eluate, then purified by further elution with *n*-hexane through a column of alumina. The column height:diameter ratios were never less than 10:1. All columns were surrounded by water jackets at 40°, and the rate of elution was always 8 ml./min.

The solvents were partially removed in a rotary evaporator at 40°. and completely in a vacuum oven at the same temperature over a dessicant.

Gas chromatography. A Pye 'Series 104' Chromatograph (Model 24) was used fitted with stainless steel columns packed with Phasecarb L on Universal support B, 85–100 mesh (Phase Separations Ltd., Cheshire). Columns were purged with argon at 325° for 48 hr before use. Samples were run at 300° isothermally also programmed from 230° to 280° at 8°/min., then maintained at the higher temperature. Carrier gas flowed at 50 ml./min. (45 lb./in.²), hydrogen, 50 ml./min. (18 lb./in.²) and air, 500 ml./min. (30 lb./in.²) were supplied to a flame ionization detector. Samples, dissolved in *n*-hexane, were injected with a 1 µl. syringe (S.G.E. Melbourne) fitted with a Chaney adapter. All columns were plugged with glass wool, since stainless steel column plugs caused spread of the sample. The recorder was a Kent Mark 3 Instrument (George Kent Ltd., Beds.; range –0.1 to 1.0 mV).

The retention volumes of the *n*-alkanes were compared with that of *n*-eicosane which was added to the samples on their second test run. At regular intervals a mixture of known *n*-alkanes (C_{16–36}) was injected for further comparisons. The relative concentrations of the hydrocarbons were calculated from the peak areas.

Reagents. Solvents used in extraction and chromatographic procedures were of Analar grade or of higher purity. Solvents of insufficient purity were redistilled. Storage was over anhydrous Na₂SO₄.

RESULTS

Analysis of lipid fractions. Table 1 shows that the average fungal lipid contents were generally higher than those in bacteria. The fungi, *Aspergillus* sp., *Trichoderma viride*, and the bacteria, *Bacillus* sp., *Mycobacterium* sp. and the Gram-negative rod, had higher lipid contents when grown on TSB. The algae, however, contained less lipid when acetate was added to the salts media. In nearly all the microbial lipids the smallest fraction was paraffinic and the largest contained polar compounds.

Gas chromatographic analyses of microbial hydrocarbons. These are presented in Table 2; unidentified peaks bear the symbol '?'. The carbon preference indices of the paraffins are given in Table 3; in the fungi and bacteria they tended to unity. The *Trichoderma viride* paraffins showed a slight C_{even} predominance especially when the fungus was grown on TSB. Algal paraffins showed a strong C_{odd} predominance except *Tetraspora gelatinosa* grown on Knop's medium with no added acetate. There was a slight tendency for bacterial paraffins from BSM to exhibit greater C_{odd} predominance, but this was not considered significant. *Arthrobacter* sp. contained the highest proportion of *n*-alkanes in the range C_{16–22}, especially if grown on TSB. The most even distri-

bution of *n*-alkanes (C_{16-32}) was in the Gram-negative rod, grown on both media, and in *Corynebacterium* sp. grown on TSB. The simplest pattern was in the *Micrococcus* sp., which consisted almost entirely of *n*-alkanes in the range C_{25-29} . There was no clear trend in the patterns of bacterial paraffins but most species showed a peak in the region C_{26-31} which was most evident in the *Bacillus* sp. and the *Mycobacterium* sp. regardless of growth medium. A minor peak was sometimes noted in the region C_{18-22} .

Table 1. *Microbial lipids*

Lipids were extracted from micro-organisms (for media see 'Methods') with chloroform + methanol (2 + 1) for 8 hr and eluted through silica gel column with *n*-hexane, benzene and methanol. All solvents removed under vacuum at 40°. Contents expressed as mg. hydrocarbon/g. dry wt organism.

	Medium	Lipid	Paraffins	Aromatics	Polar compounds
<i>Arthrobacter</i> sp.	BSM	41	9.3	1.9	10.4
	TSB	22	3.5	0.8	8.3
<i>Micrococcus</i> sp.	BSM	100	6.8	33.4	45.4
	TSB	30	3.8	0.9	10.1
Gram-negative rod	BSM	28	0.08	2.7	13.9
	TSB	126	2.4	1.2	105.4
<i>Corynebacterium</i> sp.	BSM	178	1.7	6.2	62.1
	TSB	49	10.9	2.5	22.3
<i>Bacillus</i> sp.	BSM	64	3.3	32.0	18.9
	TSB	92	0.5	0.9	35.4
<i>Mycobacterium</i> sp.	BSM	151	26.9	10.4	61.5
	TSB	193	21.4	3.2	97.1
<i>Penicillium</i> sp.	BSM	145	0.8	1.6	140
	TSB	158	0.5	1.9	95.7
<i>Aspergillus</i> sp.	BSM	101	0.6	1.1	46.2
	TSB	190	0.4	9.9	87.7
<i>Trichoderma viride</i>	BSM	43.5	7.0	3.7	29.3
	TSB	112	0.6	9.8	84.4
<i>Saccharomyces</i> sp.	BSM	178	0.4	0.8	3.5
	TSB	170	1.1	3.9	54.7
<i>Tetraspora gelatinosa</i>	1	144	28.8	23.8	81.1
	2	96	6.6	8.3	48.7
<i>Nostoc</i> sp.	3	104	5.9	30.7	88.4
	4	26	0.8	4.8	29.1

Fungal hydrocarbons showed a more stable pattern than bacteria, with a major peak at around C_{29} and a minor one in the range C_{17-20} . The exception was *Trichoderma viride* grown on BSM, which had peaks at C_{30} and C_{34} . These disappeared on TSB and an increase of an unidentified component around C_{16} and a slight peak at C_{24} were noted. These components make a major contribution towards the slight C_{even} predominance of the paraffins of this species when grown on TSB.

The algal paraffins showed a large peak at C_{17} regardless of the growth medium used. Acetate in the medium increased the C_{25} and C_{27} paraffins in *Tetraspora gelatinosa* but not *Nostoc* sp. The blue-green alga contained a simpler pattern of hydrocarbons than any of the other micro-organisms, and this did not include a peak at C_{29} .

A few hydrocarbons remained unidentified and other *n*-alkane peaks were thought, because of their shape, to be impure. These are considered in more detail in the discussion.

Table 2. *Hydrocarbon contents of micro-organisms*

Extracts of micro-organisms were examined by g.l.c. (for details of culture media, extraction and chromatography see text); hydrocarbon contents as % (w/w) total paraffin from peak areas; 't' indicates 'trace' detected, * indicates impure material, ? signifies unidentified hydrocarbon peak.

Percentage of total paraffins																							
Gram-negative rod		Arthrobacter sp.		Micrococcus sp.		Coryne-bacterium sp.		Bacillus sp.		Mycobacterium sp.		Penicillium sp.		Aspergillus sp.		Saccharo-myces sp.		Trichoderma viride		Tetraspora gelatinosa		Nostoc sp.	
BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	1	2	3	4
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	9.30	0.09	t	—	—	t	0.17	—	4.82	—	—	t	—	—	—	—	0.38	0.51	0.84	—	—	t	—
0.38	4.44	21.39	0.02	—	—	0.09	0.83	—	—	—	0.05	t	0.07	—	—	—	—	—	—	—	—	—	t
—	—	—	—	—	—	0.09	1.11	—	*1.57	—	—	0.13	0.37	—	—	—	—	—	—	t	—	—	—
3.85	10.69	1.30	0.07	0.09	—	*9.66	7.33	t	9.13	1.46	1.04	1.61	2.5	*1.7	4.98	0.10	3.88	t	—	*19.61	*30.05	71.39	75.65
—	—	0.26	—	—	—	3.77	—	—	—	—	—	3.89	—	—	—	—	4.28	—	—	—	—	—	—
5.77	6.94	0.57	0.10	0.04	1.88	*9.14	10.00	—	0.04	4.43	2.59	1.56	7.32	*3.06	*0.75	2.60	0.33	1.75	2.08	0.41	0.17	t	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4.62	3.47	0.39	0.78	0.01	—	6.90	6.00	—	0.10	0.87	5.56	1.82	1.14	*0.16	1.08	0.42	1.72	0.51	0.16	0.13	*0.81	0.06	0.09
—	—	—	—	—	—	t	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5.38	1.39	4.52	0.10	0.14	—	3.45	4.00	0.11	1.30	2.22	2.86	1.14	1.04	0.18	0.91	0.50	1.31	1.56	0.94	4.7	2.30	3.92	0.20
—	—	—	—	—	—	0.52	0.17	—	—	—	—	—	t	—	—	—	—	—	—	—	t	—	—
3.46	1.94	16.87	37.90	0.20	—	3.10	3.33	—	1.30	1.46	2.86	1.43	0.97	0.20	0.69	0.50	1.63	1.29	1.56	1.87	0.53	2.94	0.43
—	—	3.48	—	—	—	0.52	—	—	—	—	—	—	—	t	t	—	0.08	—	—	t	—	—	—
3.46	3.33	16.30	30.98	0.03	—	3.45	2.67	0.27	1.26	1.46	3.65	1.39	1.60	0.44	1.15	0.63	0.56	0.78	12.00	0.09	0.30	0.06	0.04
—	—	—	—	—	—	5.69	—	—	—	—	—	—	—	t	t	t	—	1.55	—	—	—	1.24	—
3.08	3.89	2.15	3.40	0.18	—	0.60	6.33	0.50	1.43	4.44	0.05	1.25	1.60	0.72	1.40	0.75	3.28	2.58	7.88	4.09	1.40	4.22	0.59
—	—	—	2.80	—	—	2.41	—	—	—	—	—	—	—	—	t	t	—	7.35	—	—	—	—	—
3.85	3.89	3.13	4.45	0.21	—	0.52	3.33	2.46	2.96	4.44	2.60	1.71	2.50	2.00	2.20	1.21	2.84	1.69	16.34	0.57	0.15	0.08	0.30
—	—	0.21	3.27	—	—	0.43	1.17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7.31	5.00	1.04	—	0.90	—	1.38	6.33	4.71	5.09	9.63	4.17	4.86	5.35	5.13	4.43	3.75	6.75	2.47	1.25	3.35	20.4	8.31	13.28
—	—	0.33	4.9	—	—	0.86	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	16.83	—	3.10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6.54	6.67	2.09	0.65	2.89	13.21	5.52	2.50	7.71	8.70	10.37	10.94	8.21	8.89	9.40	8.15	9.17	12.16	5.19	1.09	5.22	0.70	0.39	0.70
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9.62	7.36	7.65	1.67	45.70	11.32	17.93	6.67	32.57	13.39	17.78	10.94	12.96	14.58	15.12	13.29	15.50	11.25	3.12	1.97	13.30	28.37	4.29	4.57
7.69	7.50	6.26	1.33	32.40	39.62	7.24	8.00	15.71	14.87	20.74	28.13	13.07	16.25	16.52	16.49	20.00	11.78	5.45	2.50	11.74	2.12	0.37	0.91
10.77	8.89	6.26	1.20	0.94	30.19	7.24	6.00	13.00	13.04	11.85	15.62	14.86	15.56	17.64	17.23	19.50	15.19	8.31	3.06	15.65	5.63	0.41	1.39
—	—	—	—	0.01	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
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3.85	5.00	2.78	1.05	0.01	—	4.14	6.00	8.36	8.87	2.96	6.25	11.25	10.00	13.20	10.83	13.75	8.13	12.47	1.75	10.43	1.28	0.08	0.78
19.62	2.50	2.61	0.60	—	—	3.45	10.00	6.07	6.09	2.96	6.25	9.29	10.00	9.52	7.39	9.00	6.75	2.34	1.13	5.65	2.23	—	0.43
—	7.22	1.04	0.40	—	—	0.86	8.00	1.57	3.13	—	—	3.00	3.06	3.84	3.46	3.75	2.44	5.06	0.88	0.96	0.60	—	0.30
—	—	1.04	0.12	—	—	0.86	—	1.71	1.65	—	—	1.39	2.08	2.80	1.97	1.50	2.13	12.99	0.44	1.39	—	—	0.17
—	—	—	0.53	—	—	—	—	0.54	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
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Table 3. *Carbon preference indices derived from chromatographic analysis of microbial hydrocarbons*

	Growth medium	Carbon preference index
Gram-negative rod	BSM	1.38
	TSB	1.20
<i>Arthrobacter</i> sp.	BSM	1.17
	TSB	0.67
<i>Micrococcus</i> sp.	BSM	1.33
	TSB	0.79
<i>Corynebacterium</i> sp.	BSM	1.34
	TSB	1.24
<i>Bacillus</i> sp.	BSM	1.60
	TSB	1.09
<i>Mycobacterium</i> sp.	BSM	0.70
	TSB	1.18
<i>Penicillium</i> sp.	BSM	1.02
	TSB	1.11
<i>Aspergillus</i> sp.	BSM	1.12
	TSB	1.12
<i>Trichoderma viride</i>	BSM	0.76
	TSB	0.54
<i>Saccharomyces</i> sp.	BSM	1.03
	TSB	1.25
<i>Tetraspora gelatinosa</i>	1	1.61
	2	10.70
	3	11.56
<i>Nostoc</i> sp.	3	11.56
	4	18.17

DISCUSSION

Lipids made up between 2 and 20 % of the bacterial dry weights, the highest levels being found in *Mycobacterium* sp. The levels were similar to those reported by Albro & Huston (1964), Davis (1967) and Davis (1968). There was good correlation between published data and the paraffin contents of the bacteria (Han *et al.* 1968; Tornabene, Bennet & Oró, 1967).

A variety of bacterial hydrocarbon patterns was noted and only certain general trends will be discussed here. The range of *n*-alkanes (C_{16-36}) was similar to those reported by authors mentioned below. The minimum at C_{18-21} reported by Clarke (1966) was only evident in the *Bacillus* sp. grown on BSM; indeed a minor peak at this range was noted in some species. A modest maximum was almost always present at C_{27-30} (Albro & Huston, 1964; Davis, 1968). Tornabene, Bennet & Oró (1967) reported a more even hydrocarbon distribution when *Sarcina lutea* was grown on complex rather than simple medium and although this was true of the Gram-negative rod in this study, it was not observed in the other species. The same authors reported a C_{odd} predominance on a simple medium but not on Trypticase Soy Broth; this trend was not observed in the organisms used in this investigation.

Higher carbon preference indices were sometimes obtained when bacteria were grown on BSM but the values were not considered to be significantly different from those on TSB.

The lipid and hydrocarbon contents of the fungal and algal species were similar to those reported by Han *et al.* (1968) and Davis (1967) and so was the range of paraffins.

The patterns were less variable than those in bacteria but were not always similar to those reported by these authors. The *Saccharomyces* sp. did not have a peak at C₂₂ unlike *Debaryomyces hansenii* (Merdinger & Devine, 1965). The patterns of hydrocarbons in three of the fungi (peaks at C₂₇₋₂₉) were similar to those reported in the spores of *Ustilago* sp. and *Sphacelotheca* sp. (Oró *et al.* 1966).

The increase in C₂₅ and C₂₇ components of *Tetraspora gelatinosa* in the presence of acetate was similar to that reported by Patterson (1967) in *Chlorella vulgaris*, grown chemo-organotrophically, although in the latter organism the hydrocarbons were monoenes. The predominant C₁₇ paraffin was present in both algae, being thus in agreement with Han *et al.* (1968) and Oró *et al.* (1967). The suspect purity of the C₁₇ paraffin in *T. gelatinosa* suggests the presence of the corresponding alkenes.

The carbon preference indices in fungi agreed with those in the literature. A value of around unity was also obtained when *Tetraspora gelatinosa* was grown on CO₂ as sole carbon source as with *Chlorella vulgaris* (Patterson, 1967) but not when acetate was added to the medium. *Nostoc* sp. hydrocarbons exhibited a strong C_{odd} predominance on both media.

The hydrocarbons labelled '?' in the tables were thought to be impure. From the retention volume data (Albro & Huston, 1964) with a similar column and published results (Oró *et al.* 1967; Han *et al.* 1968; Patterson, 1967; Tornabene, Gelpi & Oró, 1967), some components detected immediately before or with the *n*-alkane may be the corresponding *n*-alkanes.

The analyses of microbial hydrocarbons are in general agreement with those of Han *et al.* (1968). In algae the major component is a C₁₇ hydrocarbon and the majority have chain lengths shorter than C₂₀. In bacteria and fungi the greater proportion have chain lengths greater than C₂₀. This does not, however, agree with the results of Oró *et al.* (1967) and Tornabene, Gelpi & Oró (1967), who reported lower hydrocarbons in certain bacteria (*Vibrio marinus*) and unsaturated, methylbranched paraffins in *Sarcina lutea* ATCC 535. Albro & Huston (1964) and Tornabene, Gelpi & Oró (1967) obtained differing results with *S. lutea*. Obviously microbial hydrocarbon patterns depend on growth media, growth conditions and analytical methods. Standardized procedures for growth, extraction and analysis of microbial hydrocarbons should be adopted. Since the growth medium used influences the hydrocarbons produced any attempt to correlate such results with possible geological roles of micro-organisms (Han *et al.* 1968) should take account of the substrates available in the field.

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