

Bacterial Lipids

1. Lipid Constituents of a Moderately Halophilic Bacterium

MOIZ F. MULLAKHANBHAI^{a*} and GEORGE W. FRANCIS^{b**}

^aDepartment of Biochemistry, and ^bDepartment of Organic Chemistry, Norway Institute of Technology, University of Trondheim, N-7034 Trondheim-NTH, Norway

The lipid constituents of a bacterium isolated¹ from Dead Sea sediment are examined. The lipid is shown to be largely terpenoid and although differing quantitatively from that of the previously investigated²⁻⁵ extreme halophiles is qualitatively similar. The polar lipid is largely phosphate esters of 1,2-di-*O*-(dihydrophytyl)-glycerol which occurs free in the non-polar lipid. A symmetrical tetrahydro-squalene lacking the double bonds of the terminal prenyl units is the major component of the non-polar lipid. In addition, bacterial vitamin MK-8 is present. The major carotenoid of this bacterium corresponds to a formula C₅₀H₇₄O₄ and has two tertiary hydroxyl groups and two ether oxygen atoms. Bacterioruberin, the typical carotenoid of halobacteria,⁶⁻⁸ represents 20 % of the total carotenoid content.

The lipids of halophilic bacteria have been investigated in an elegant series of papers by Kates *et al.*²⁻⁵ Kates examined the lipids of seven extreme halophiles and compared these with those of a number of moderate halophiles and non-halophiles.² Extreme halophiles were characterised by the occurrence of phosphate esters of 1,2-di-*O*-(dihydrophytyl)-glycerol (*1*). These compounds were found to be absent in the other bacteria investigated. In addition, it was found that fatty acid derivatives were present in all but the extreme halophiles.

Squalene (*2*) was shown³ to be a major constituent of the non-polar lipid of *H. cutirubrum*. Mass spectra and nuclear magnetic resonance spectra were used to demonstrate the presence of a dihydrosqualene with one terminal double bond reduced and a tetrahydrosqualene with one internal and one terminal double bond reduced as minor components. In the same investigation the presence of bacterial vitamin MK-8 (*3*) was described.

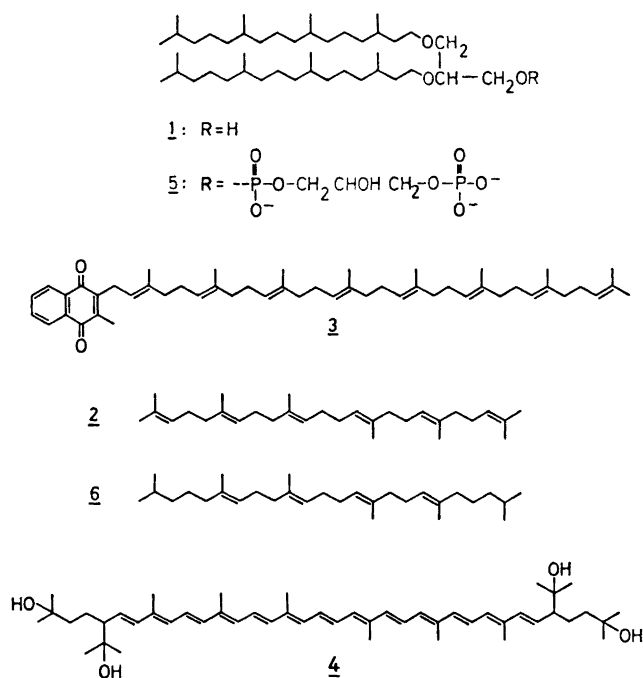
Early work on the carotenoid content of halobacteria has been reviewed by Liaaen-Jensen.⁶ Recently Kelly *et al.*^{7,8} have described the structure of

* Present address: Dept. of Microbiology, Haryana Agric. University, Hissar, Haryana, India.

** Present address: Dept. of Chemistry, University of Bergen, N-500 Bergen, Norway.

bacterioruberin (4), the typical carotenoid of halobacteria, and detailed the carotenoid composition of *H. salinarium*.⁸ The carotenoids of this last bacterium were shown to have C₅₀-skeletons for the most part.

A bacterium has recently been isolated in our department of biochemistry from Dead Sea sediment.¹ This bacterium is characterised physiologically by its fitness for life in an ionic environment like that found in the Dead Sea. It has a moderate requirement for sodium chloride as compared to the extremely halophilic *halobacteria* and a very high requirement for and tolerance of magnesium chloride. It was thus of interest to see to what extent the lipid of this somewhat unique bacterium corresponded to that of the previously investigated extreme halophiles.²⁻⁸



RESULTS AND DISCUSSION

Growth and harvesting of the organism, and preparation of the total lipid have been described elsewhere.¹ Examination of the total lipid by infrared absorption spectroscopy (Fig. 1a) revealed neither carbonyl frequencies at 1700–1750 cm⁻¹ nor broadening of absorption in the region 2500–2800 cm⁻¹. This was similar to the position found in the lipid of extreme halophiles² and in contradistinction to the lipids of moderate and non-halophilic bacteria. Absorptions assignable to the presence of phosphate and hydroxyl groups were obvious in addition to a number of frequencies assignable to C–H bondings.

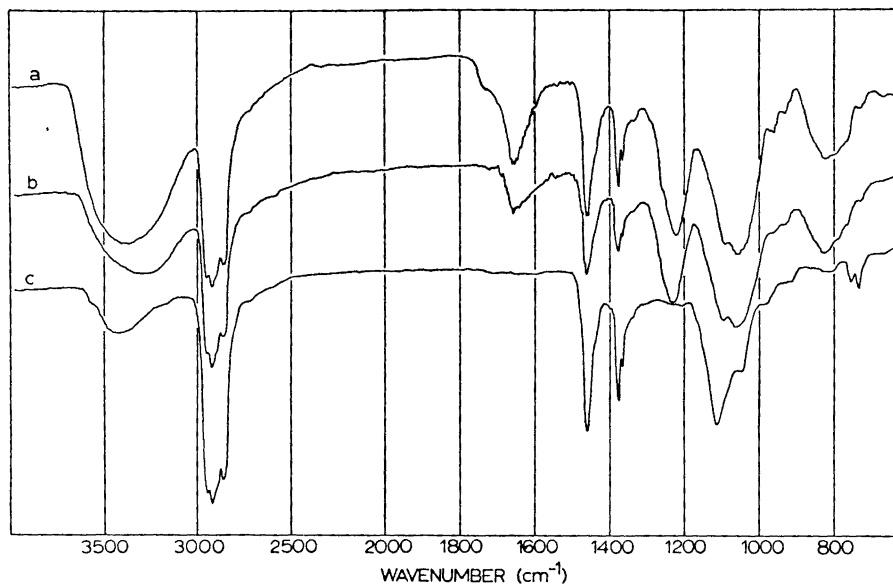


Fig. 1. Infrared spectra (liquid, film) of (a) total lipid, (b) polar lipid, and (c) 1,2-di-(dihydrophytyl)-glycerol (1).

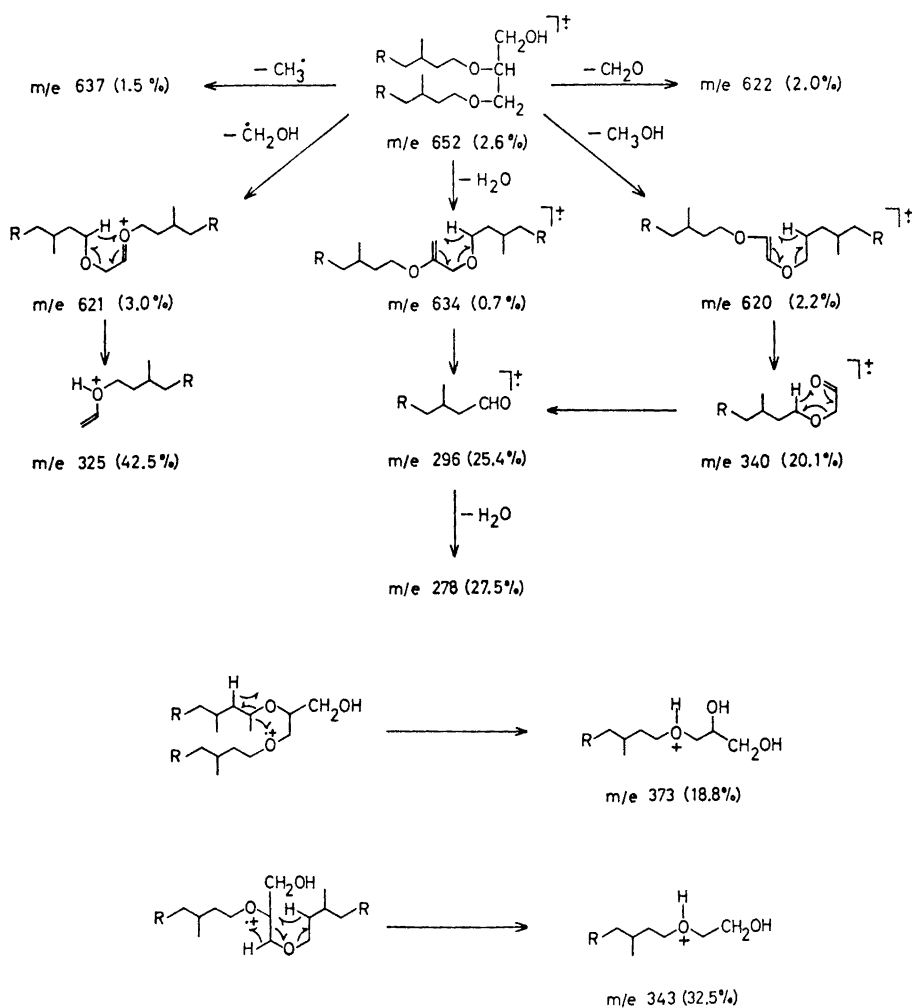
Polar lipid

The total lipid was extracted overnight with cold acetone and the insoluble material (78 %) re-examined by infrared spectroscopy (Fig. 1b). Comparison of this spectrum with that of the total lipid showed no major alterations. The polar lipid was now hydrolysed by refluxing with 0.7 N methanolic hydrochloric acid. This resulted in the hydrolysis of the phosphate linkages and thin layer chromatography showed one major component (*ca.* 90 %) in the epiphase on extraction with petrol. The aqueous products were not further examined.

Preparative thin layer chromatography of the major hydrolysis product in two solvent systems ($\text{H}_2\text{O}/\text{MeOH}/\text{CHCl}_3$, 1/10/90, $R_F = 0.93$; $\text{AcOH}/\text{EtOEt}/\text{petrol}$, 1/10/90, $R_F = 0.25$) yielded a pure compound. The infrared absorption spectrum (Fig. 1c) showed that this compound contained hydroxyl and ether functionalities but no multiple bonds. The nuclear magnetic resonance spectrum confirmed this latter in that it showed only signals at 6.4 τ (broad), 8.8 τ (mult.), and 9.14 τ (doublet, $J = 6$ cps). The mass spectrum showed a molecular ion at m/e 652 and this and integration of the nuclear magnetic resonance spectrum allowed tentative identification of the compound with 1,2-di-*O*-(dihydrophytyl)-glycerol (1). Additional evidence was found in the mass spectral fragmentation.

The ions observed in the molecular ion region were all relatively unintensified as would be expected from the proposed polyol structure. In addition to the molecular ion, other moderately intense ions were observed at $M - 15$, $M - 18$,

M-30, M-31 and M-32 and these could be rationalised as being due to loss of a lateral methyl group, water, formaldehyde, a CH_2OH radical, and methanol, respectively. The last three of these losses were particularly significant in that it would be difficult to explain their derivation from the isomeric 1,3-disubstituted glycerol. A series of very intense ions in the central part of the mass range provided conclusive evidence for the proposed identification in that all could be derived by logical rearrangement of the molecular ion or by McLafferty rearrangement⁹ of other ions in the molecular ion region. The lower part of the spectrum was dominated by ions of the series $\text{C}_n\text{H}_{2n+1}$ as would be expected from any isoprenoid structure of the type proposed.



Scheme 1. Derivation of significant ions in the mass spectrum of 1,2-di-(dihydrophytyl)-glycerol (I). The relative intensities of the ions are given in brackets.

These last ions increased steadily in intensity from $n=15$ (5.1 %) to the base peak ion at $n=4$. Possible derivations for the significant ions in the spectrum are shown in Scheme 1, although alternative paths may be written in some cases.

Acetylation of the free alcohol (*I*) with acetic anhydride-pyridine gave a monoacetate with molecular ion at m/e 694 and a much simplified mass spectrum. In the molecular ion region the most intense ion occurred at $M-60$ (4.2 %) and was attributed to loss of a molecule of acetic acid from the molecular ion and both M (0.8 %) and $M-15$ (0.7 %) ions were much less intense. The directive effect of the acetyl group was also seen in the fact that only three prominent ions now occurred in the central part of the mass spectrum and all could be explained as being directly formed from the molecular ion. The most intense of these at m/e 383 was indeed the base peak ion and could be readily explained as being due to cleavage of one of the glycerol-ether oxygen linkages, a process not important in the free alcohol (*I*). The m/e 397 (8.3 %) ion corresponded to fission of the C_1-C_2 bond of the glycerol moiety and that at m/e 415 (3.4 %) was analogous to the m/e 373 ion in the parent compound.

The evidence detailed above was considered sufficient to identify the main constituent of the saponified lipid as 1,2-di-*O*-(dihydrophytyl)-glycerol (*I*). In view of the presence of phosphate linkages before saponification it is assumed that the complete structure of the natural material was similar to the phosphatide (*5*) described by Kates *et al.*⁵ as the main component of the polar lipid of *H. cutirubrum*.

Acetone-soluble lipid

The acetone-soluble (non-polar) lipid could be separated into components in two different ways: either by thin layer chromatography using a polar solvent containing acetic acid or by initial column chromatography on cellulose with ether/petrol mixtures followed by thin layer chromatography using less polar solvents lacking acetic acid. The first procedure, though highly satisfactory for other components, resulted in the destruction of the carotenoid pigments.

When the first described method was used some 20 % of the total acetone-soluble lipid was recovered from a highly polar fraction which contained the largely destroyed carotenoids together with some fluorescent material. This fraction was not further studied. Three major components were separated and these represented 68 % of the total (Table 1).

Fraction 1 was rechromatographed on a thin layer plate using petrol as eluent. It contained only one component which was somewhat less polar than squalene (R_F -values: *Fraction 1* = 0.78, squalene = 0.55). As would be expected from the polarity the infrared spectrum showed no absorbances other than those attributable to a hydrocarbon. The mass spectrum showed a molecular ion at m/e 414 and this corresponded to a molecular formula of $C_{20}H_{34}$ as would be found for a tetrahydrosqualene. Total lack of ions at $M-69$ suggested that the missing double bonds were those normally found in the terminal prenyl units. In addition, it would be necessary to assume multiple hydrogen transfers to explain some of the more intense ions in the upper part

Table 1. Thin layer chromatography of the acetone-soluble lipid. Solvent AcOH/EtOEt/Petrol, 1/10/90; Kieselgel G layer; UV indicates active in ultraviolet light; VIS is visible colour on plate; I₂ indicates noticeable staining with this reagent.

Fraction	Identity	R _F -value	UV	VIS	I ₂	% of total
1	Tetrahydro-squalene (6)	0.99	no	no	yes	43
2	Dihydro-MK-8	0.88	yes	no	no	2
3	Bact. vitamin MK-8 (3)	0.72	yes	yes	yes	13
4	—	0.56	yes	no	no	4
5	—	0.43	yes	no	no	6
6	Glycerol diether (1)	0.25	yes	no	yes	12
7	Decomp. carotenoids <i>inter alia</i>	0.00–0.13	yes	red-yellow	no	20

of the spectrum were any other structure assumed (Fig. 2). However, much more conclusive structural evidence was obtained from the nuclear magnetic resonance spectrum (Fig. 3) which showed signals at the same τ -values as reported by Tornabene *et al.*³ for a tetrahydro-squalene isolated from *H. cuti-rubrum*. Integration showed that there existed a 1/1 ratio of the methyl groups attached to fully saturated C-atoms and those attached to double bonds. This observation can only be accounted for by the structure 6. Tornabene³ found a ratio close to that expected by theory (5/3) for a structure with one internal and one terminal double bond reduced. A further difference exists between the two compounds in that that author recorded an M–69 ion in the mass spectrum and this is good evidence for a structure with at least one terminal double bond present.

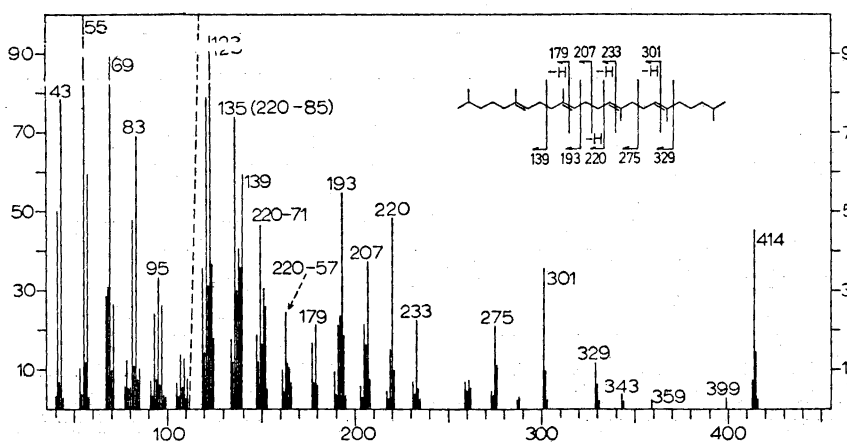


Fig. 2. Mass spectrum of tetrahydro-squalene (6).

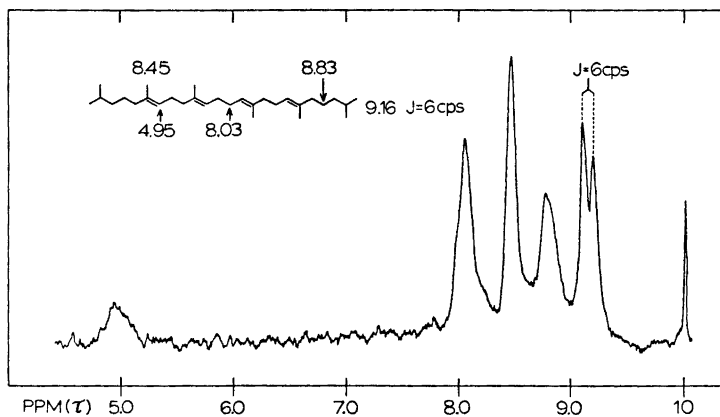


Fig. 3. Nuclear magnetic resonance spectrum of tetrahydroqualene (6).

Fraction 3 was yellow on the plate and highly active in ultraviolet light. The previous investigations had shown that bacterial vitamin MK-8 (3) was present in a related bacterium³ and when the spectrum of *Fraction 3* was recorded and found to be identical (Fig. 4) further evidence was immediately sought to confirm identity. The ultraviolet absorption spectrum of *Fraction 3*

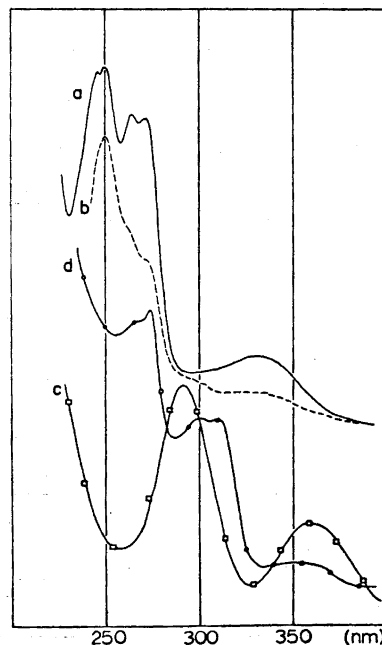
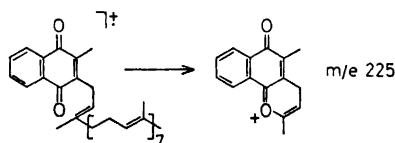


Fig. 4. Ultraviolet spectra in ethanol of (a) *Fraction 3*, (b) *Fraction 3* after reduction with NaBH_3 , (c) *Fraction 5*, and (d) *Fraction 6* of the acetone-soluble lipid.

was changed on reduction with sodium borohydride in the way previously shown to occur³ with genuine bacterial vitamin MK-8 (3) on similar treatment. The mass spectrum showed a molecular ion at m/e 716 and a prominent ion at m/e 225 (49 %) assignable to the ion depicted below.¹⁰ A series of ions corresponding to loss of fragments of mass $68n + 1$ ($n = 1 - 7$) was due to cleavage of the doubly allylic single bonds in the side chain with retention of charge on the naphthoquinone residue. *Fraction 2* was thus identified as bacterial vitamin MK-8 (3).



Fraction 2 was isolated in microgram quantities and was contaminated with low molecular weight impurities. The mass spectrum, the only evidence available, showed a molecular weight of 718 and a prominent ion at m/e 225 as above. Since this last ion has been claimed¹⁰ to be of diagnostic value in confirming the presence of a 2-methyl-1,4-naphthoquinone system bearing a 3-isoprenoid chain where at least the first isoprenoid unit contains unsaturation this structural feature is assumed. Thus this fraction is presumed to be a dihydro-MK-8 having a double bond remote from the naphthoquinone system reduced. Such a compound has already been isolated by Scholes and King¹¹ from a *Corynebacterium*.

The minor components *Fractions 4 and 5* both showed long wavelength ultraviolet absorption although the spectra were very different in appearance (Fig. 4). It is supposed that they are both quinonoid in nature but no further evidence is at present available.

Fraction 6 appeared from its mass spectrum to be identical with 1,2-di-*O*-(dihydrophytyl)-glycerol and co-chromatography on the two solvent systems used was taken as conclusive. It should be noted, however, that at least some quantity of an ultraviolet active compound was present as an impurity.

Carotenoids

In order to obtain an analysis of the carotenoid pigments the total acetone-soluble lipid was first chromatographed on a cellulose column using ether/petrol mixtures as eluting solvent. Four coloured zones were thus obtained and these were then re-chromatographed on thin layer plates using appropriate solvents (see Table 2). This procedure showed that *zones A, C, and D* were single pigments while *zone B* was a complex mixture. The three single component zones and the major component of *zone B* (*B3* and *B4*) all showed electronic absorption spectra like those found in aliphatic carotenoids with undecaene or longer conjugated systems. The position of the main maxima of *zones A* and *B3/4* were indeed indicative of an undecaene chromophore

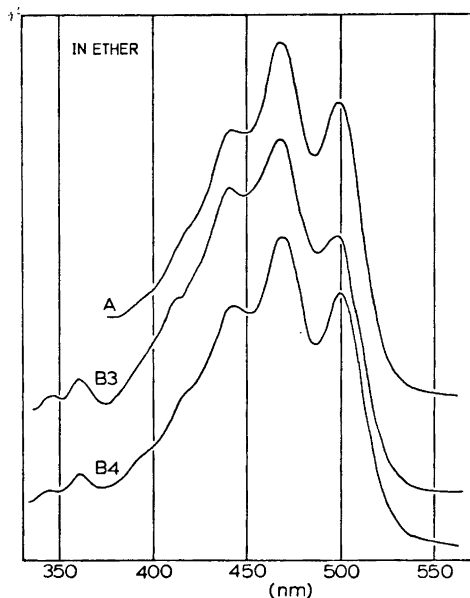


Fig. 5. Visible absorption spectra of the carotenoid zones A, B3, and B4.

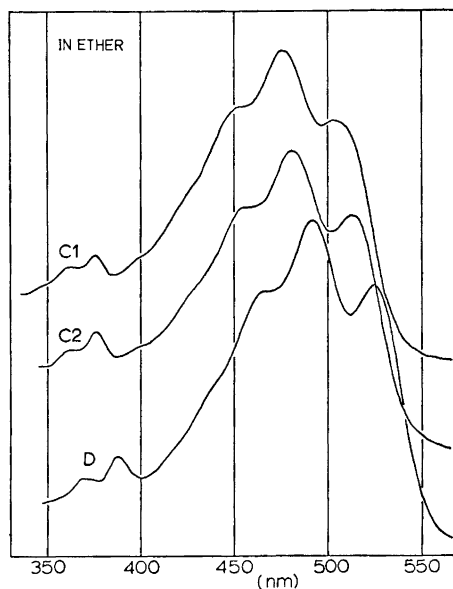


Fig. 6. Visible absorption spectra of the carotenoid zones C1, C2, and D.

(Fig. 5) while zone C with main maximum at 481 nm and zone D with main maximum at 492 nm could be assigned dodecaene and tridecaene chromophores, respectively (Fig. 6). It should be noted that all major zones appeared to contain *cis* isomers as judged by the presence of *cis*-peaks at *ca.* 360 nm. It was on the basis of their sharing the same *cis*-peaks that zones B3 and B4,

Table 2. Carotenoid pigments of the new moderate halophile. Visible maxima marked * are regarded as unreliable.

Fraction	Polarity on TLC plates (R_F in acetone/petrol)				Visible abs. max. λ_{max} (nm)	%
	5 %	10 %	20 %	50 %		
A	v. non-polar	—	—	—	499, 469, 442 (yellow)	7.8
B1	0.5 <i>ca.</i>	—	—	—	460, 433, (415), 363 (yel)	2.6
B2	0.39	—	—	—	460 (red)	1.6
B3	0.25	0.43	—	—	499, 469, 440, 360, 345 (or)	5.0
B4	0.21	0.39	—	—	500, 469, 443, 361, 345 (or)	13.2
B5	0.15	0.32	0.57	—	450 (red)	5.1
B6	—	0.2 <i>ca.</i>	—	—	450 (red)*	2.7
B7	—	0.10	0.34	—	440 (red)*	2.0
C1	—	—	0.17	—	504, 477, (452), 376, 361 (red)	18.4
C2	—	—	0.15	—	513, 481, (454), 376, 361 (red)	21.4
D	—	—	0.01	0.48	525, 492, 465, 388, 369 (red)	20.2

and *C1* and *C2*, were assigned as *cis-trans* isomers. Together the above pigments, *A*, *B3/4*, *C*, and *D*, represent 86 % of the total carotenoid and must therefore be taken as typical carotenoids of this bacterium. This finding is in agreement with previous studies on halophilic bacteria which have shown⁶⁻⁸ a high incidence of carotenoids with aliphatic chromophores.

Further work on the carotenoid pigments was hampered by the occurrence of small amounts of fluorescent contaminants which were not readily separable. In particular, this hindered mass spectral analysis which was the only method available to study the small quantities of pigment present. Only pigments *C* and *D* were thus further studied.

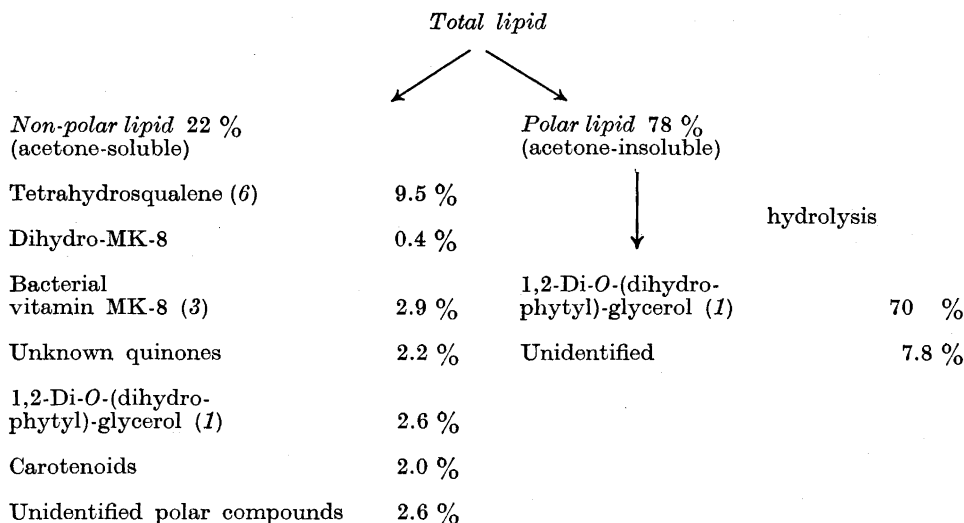
Pigment *C* gave a molecular ion at m/e 738 which suggested a formula of $C_{50}H_{74}O_4$. The assignment of the molecular ion was supported by the observation of $M-92$ and $M-106$ ions.¹² Ions explicable as derived by loss of two molecules of water were recorded and in some cases such losses were observed as part of more complex fragmentation. In addition, a relatively intense $M-58$ ion could be attributed to the presence in the molecule of at least one tertiary hydroxyl group in an environment where two methyl groups and the hydroxyl group were attached to the same C-atom and the remaining bond to this atom was suitably activated.¹³ Pigment *C* did not yield an acetate under standard acetylating conditions¹⁴ and thus contained neither primary nor secondary hydroxyl functions. A di-(trimethylsilyl) ether was obtained by the standard method¹⁵ as evidenced by mass spectral analysis of the product: $M=882$, $M-72$, $M-90$, $M-92$, $M-106$ and m/e 131. This last ion, the base peak, provided further support for the situation of the tertiary hydroxyl groups in that it is normally found in systems having present the system $R-CMe_2OSiMe_3$. Pigment *C* was recovered unchanged after prolonged treatment with LAH.

The above evidence leads to the conclusion that pigment *C* contains two ether oxygen atoms, two tertiary hydroxyl groups and a dodecaene chromophore. A similar pigment has previously been described as a minor component of the bacterium *H. salinarium* by Kelly *et al.*⁸ (*loc. cit.*: Natural C_{50} -diol (19)) and possible identity must be considered.

Pigment *D* had visible absorption maxima compatible with a tridecaene chromophore. Mass spectrometry showed a molecular weight of 740. Acetylation failed and the compound gave a tetra-(trimethylsilyl) ether ($M=1028$, $M-90$, $M-92$, $M-106$, m/e 131). Co-chromatography on thin layer plates of the alcohol *D* with genuine bacterioruberin and of the per-trimethylsilyl ether with that obtained from bacterioruberin was taken as evidence of identity of pigment *D* with bacterioruberin (4).

CONCLUSION

The quantitative relationship between the components of the total lipid of the new moderate halophile¹ is indicated in Scheme 2. Examination shows that the lipid is similar in content to that of previously investigated extreme halophiles²⁻⁸ though quantitative differences exist. In particular it should be noted that as in the extreme halophiles no trace of fatty acid derivatives was found. Further work on the quinones and carotenoids is projected.



Scheme 2. Constituents of lipid of new moderate halophile.

EXPERIMENTAL

Physical data for compounds are given only when not presented in the text.

Materials and methods. Solvents and reagents not of analytical grade were purified as follows: petrol (petroleum ether b.p. 40–60°) and acetone by distillation, ether by elution from an alumina column. Thin layer plates were 0.5 mm in thickness and prepared from Merck Kieselgel G, Art. 7731. Visualisation of spots on thin layer plates was with ultraviolet light, iodine vapour, Rhodamine 6G, ceric sulphate or water as appropriate. Cellulose columns were of Schleicher and Schüll cellulose powder No. 124. Infrared spectra were recorded as thin films on a Perkin Elmer 257 grating spectrometer and ultraviolet and visible absorption spectra obtained on a Coleman-Hitachi 124 spectrophotometer. Nuclear magnetic resonance spectra were recorded in deuteriochloroform solution on a Varian A-60A instrument and mass spectra determined on an AEI MS 902 mass spectrometer with ion source temperature in the range 200–250°.

Total lipid was prepared as described elsewhere.¹ Infrared spectrum (see also Fig. 1a): 3300 (bonded OH), 2960 (CH₃), 2930 (CH₂), 2870 (CH), 1660 (POH), 1465 (CH₂), 1380 and 1370 (CH₃), 1245 (P=O), 1100 and 1070 cm⁻¹ (P–O–C and O=P–O⁻).

Solvent partition of total lipid was carried out by allowing the total lipid (56 mg) to stand overnight at 0–4° with acetone (50 ml). The acetone extract was taken to dryness and gave a red oil (12.5 mg, 22%). The insoluble fraction (43.5 mg, 78%) was taken up in chloroform and represented the polar lipid.

Hydrolysis of the polar lipid (42.5 mg) was carried out by refluxing⁴ with methanolic HCl (5 ml, 0.7 N) for 4 h. A few ml of water were then added and the resulting single phase extracted with small portions of petrol. The petrol extracts were pooled and washed with water to neutrality. The petrol extract was taken to dryness on the rotary evaporator and azeotropic distillation with methanol and benzene furnished a water-free product (32 mg, 74%).

1,2-di-O-(dihydrophytyl)-glycerol (I) had infrared spectral absorption at 3450 (bonded OH), 2960 (CH₃), 2930 (CH₂), 2870 (CH), 1465 (CH₂), 1380 and 1370 (CH₃), 1120 cm⁻¹ (C–O–C). Nuclear magnetic spectrum had resonances at 4.55 (ca. 9H, H–C–O), 7.88 (ca. 4OH, sat. CH₂) and 9.12 τ (ca. 3OH, doublet, J = 6 cps, H–C–CH₃). Important ions in the mass spectrum are given in Scheme 1.

Tetrahydro-squalene (6) had infrared absorptions at 2960 (CH₃), 2935 (CH₂), 2870 and 2860 (CH), 1460 (CH₂), 1385 and 1370 cm⁻¹ (CH₃). The nuclear magnetic resonance and mass spectra are given in Figs. 3 and 2, respectively.

Bacterial vitamin MK-8 (3) had ions at the following *m/e* values in the mass spectrum: 716 (22 %), 701 (0.6 %), 647 (0.4 %), 579 (0.7 %), 511 (0.7 %), 443 (0.9 %), 375 (1.3 %), 308 (3.7 %), 307 (4.0 %), 265 (2.1 %), 225 (49 %), 187 (9.8 %), 81 (51 %) and 69 (100 %). The ultraviolet spectrum is given in Fig. 4. Reduction was carried out with excess sodium borohydride in ethanol solution and the ultraviolet spectrum of the reduced form is given in Fig. 4.

Dihydro-MK-8 showed the following ions in the mass spectrum: *m/e* 718 (2.8 %), 716 (2.4 %), 225 (13 %), 85 (73 %), 71 (59 %), 69 (78 %), 57 (100 %), 55 (57 %) and 43 (65 %).

Pigment C had ions in the mass spectrum at *m/e* 738 (M, 4.1 %), 720 (M-18, 2.8 %), 704 (M-18-16, 0.8 %), 702 (M-18-18, 0.8 %), 680 (M-58, 1.0 %), 662 (M-58-18, 0.6 %), 646 (M-92, 1.2 %), 632 (M-106, 3.7 %), 630 (M-92-16, 0.7 %), 628 (M-92-18, 0.8 %), 614 (M-106-18, 2.5 %), 596 (M-106-18-18, 0.7 %), 69 (75 %) and 43 (100 %).

Pigment C di-trimethylsilyl ether had ions at *m/e* 882 (M, 1.4 %), 810 (M-72, 0.12 %), 792 (M-90, 0.23 %), 790 (M-92, 0.28 %), 776 (M-106, 0.95 %) and *m/e* 131 (100 %).

Bacterioruberin (4) and *per-trimethylsilyl ether* gave mass spectra in agreement with those previously reported.⁸

Visible absorption spectra of the major carotenoids are given in Figs. 5 and 6.

Acknowledgements. We wish to thank Professor H. Larsen who initiated this work for his valuable guidance throughout. Our sincere thanks are also due to Professor S. Liaaen-Jensen for helpful criticism of the manuscript. We acknowledge financial support from the *Norwegian Agency for International Development* (to M. F. M.) and from *Norges tekniske hogskole* (to G.W.F.).

REFERENCES

1. Mullakhanbhai, M. F. and Larsen, H. *To be published.*
2. Kates, M., Palameta, B., Joo, C. N., Kushner, D. J. and Gibbons, N. E. *Biochemistry* **5** (1966) 4092.
3. Tornabene, T. G., Kates, M., Gelpi, E. and Oro, J. *J. Lipid. Res.* **10** (1969) 294.
4. Sehgal, S. N., Kates, M. and Gibbons, N. E. *Can. J. Biochem. Physiol.* **40** (1962) 69.
5. Kates, M., Yengoyan, L. S. and Sastry, P. S. *Biochim. Biophys. Acta* **98** (1965) 252.
6. Liaaen-Jensen, S. *Kgl. Norske Videnskab. Selskabs Skrifter* **1962** No. 8.
7. Kelly, M. and Liaaen-Jensen, S. *Acta Chem. Scand.* **21** (1967) 2578.
8. Kelly, M., Norgård, S. and Liaaen-Jensen, S. *Acta Chem. Scand.* **24** (1970) 2169.
9. Budzikiewicz, H., Djerassi, C. and Williams, D. H. *Mass Spectrometry of Organic Compounds*, Holden-Day, San Francisco 1967.
10. Das, B. P. and Lederer, E. In Burlingame, A. L. *Topics in Organic Mass Spectrometry*, Wiley-Interscience, New York 1970.
11. Scholes, P. B. and King, H. K. *Biochem. J.* **97** (1965) 754.
12. Enzell, C. R., Francis, G. W. and Liaaen-Jensen, S. *Acta Chem. Scand.* **22** (1968) 1054.
13. Enzell, C. R., Francis, G. W. and Liaaen-Jensen, S. *Acta Chem. Scand.* **23** (1969) 727.
14. Aasen, A. J. and Liaaen-Jensen, S. *Acta Chem. Scand.* **20** (1966) 1970.
15. McCormick, A. and Liaaen-Jensen, S. *Acta Chem. Scand.* **20** (1966) 1989.

Received July 26, 1971.