The Membrane Lipids of *Halobacterium halobium*

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(Received 7 June 1968)

The lipid content of the cell membrane of *Halobacterium halobium* increased from about 15% to 21% during exponential growth of the organism. Total lipid phosphorus more than doubled during the growth cycle. The mixture of membrane lipids from stationary-phase organisms was similar to lipid mixtures from whole cells of other halobacteria inasmuch as 80% of the lipid phosphorus occurred in a diether analogue of phosphatidylglycerophosphate and an additional 7.5% occurred in the ether analogue of phosphatidylglycerol. The lipid mixture was more complex than those reported for other halophils, however, 12 components being recognized in the acetone-insoluble fraction and 17 in the acetone-soluble fraction. There were major changes in the proportions of some minor components of the acetone-insoluble fraction during a growth cycle. Three nitrogenous lipids were recognized in the acetone-insoluble fraction, but all were present in relatively low proportion. One, which was not a phospholipid, contained a bound peptide. Of the 17 acetone-soluble compounds, 15 were pigments. The major carotenoids were α - and β -bacteriorubrin. The carotenoid pigments occurred at maximal concentration after 6–7 days' growth.

In recent years many generalized and hypothetical structural models have been proposed for lipoprotein membranes. The type of evidence on which such models are based varies, but in no case has the chemistry of a membrane been adequately understood. There is substantial information about membrane lipids since, for technical reasons, lipids are usually easier to study than the corresponding proteins.

The solubility in water of the membrane of *Halobacterium halobium* (Brown, 1963), in addition to facilitating physicochemical studies (Brown, 1965; Brown & Netschey, 1967), offers the possibility of fractionating and analysing the (lipo)proteins without the complication of additives such as detergents. This paper describes the membrane lipids of *H. halobium* and variations in the lipid composition of the membrane during a growth cycle of the organism. The lipids from whole cells of a similar organism, *Halobacterium cutirubrum*, were studied in detail by Sehgal, Kates & Gibbons (1962). Less-detailed comparative analyses have also been made of the lipids from whole cells of several salt-tolerant and halophilic bacteria, including two strains of *H. halobium*. The lipids from the two strains of *H. halobium* closely resembled those of *H. cutirubrum*, and it was expected that the membrane lipids of our strain would also be generally similar to those of the other halophils.

The present investigation was necessitated, however, by the species and strain differences and because we are concerned solely with the membrane lipids. The expected similarities were encountered, but the membrane lipids of *H. halobium* proved to be a more complex mixture than the lipids of *H. cutirubrum*.

MATERIALS AND METHODS

Materials. All solvents and reagents were commercial A.R.-grade preparations. Solvents were distilled before use.

Methods. *H. halobium* was grown in 1.5l. shake cultures at 30° in the medium described by Sehgal & Gibbons (1960). Except in experiments dealing specifically with age of culture the organisms were harvested at the end of the exponential phase (6 days); unless a culture age is specified, analyses refer to material obtained from 6-day cultures. For the investigation of effects of culture age, each flask was inoculated with a culture (40ml.) of the organism in the mid-exponential phase of growth. At specified times the turbidity of the culture contained in one flask was measured at 680m μ and the organisms were harvested by centrifugation. Labelled membrane preparations were obtained by including [³⁵S]sulphate or [³²P]orthophosphate (0.5mc/1.5l.) in the culture medium. Membranes were prepared by osmotic lysis of the organisms in 0.02M-MgCl₂ (Brown, Shorey & Turner, 1965). This method gives a preparation consisting predominantly of membranes; the proportion of complete envelopes in such preparations is normally less than 1%.

Lipoprotein solutions were freeze-dried after dissolution of the membranes by dialysis against water at 4°. Lipids were extracted from the dried membrane preparations with chloroform-methanol (2:1, v/v) until there was no visible pigmentation of the protein fraction. The lipids were then concentrated under N₂, filtered through sintered glass, dried under N₂ and redissolved in approx. 1 ml. of chloroform-ethanol-water (80:20:3, by vol.). The solution was passed through a column (18 cm. × 1 cm.) of Whatman CF11 cellulose (Lea & Rhodes, 1953). The resulting eluate was evaporated to dryness and redissolved in a small volume of chloroform. The acetone-insoluble lipids were precipitated by diluting the chloroform solution with 10 vol. of cold acetone. This procedure was repeated and both the acetone-insoluble and acetone-soluble fractions were stored in a convenient volume of chloroform-methanol (2:1, v/v) at -20° in the dark until required. For experiments designed to show the presence of a lipopeptide, acetone-insoluble samples were purified further by washing them three times with 2 vol. of each of 0.2 M-sodium phosphate buffer, pH 7.0, 0.1 N-HCl and water, as well as by passage over Sephadex G-25 according to the method of Wells & Dittmer (1963).

For thin-layer chromatography, silica gel G (E. Merck A.-G., Darmstadt, Germany) plates, 20 cm. × 20 cm. × 0.25 mm., were prepared as described by Mangold (1961); 6.6% of (NH₄)₂SO₄ was included to prevent tailing during chromatography of the acetone-insoluble lipids. Plates were activated at 105° for 16 hr. before use. Acetone-insoluble lipids were chromatographed in chloroform-methanol-water (65:25:4, by vol.) and pigments in light petroleum (b.p. 40-60°)-acetone-diethyl ether-acetic acid (70:20:10:1, by vol.). Lipid equivalent to 25-50 µg. of P was applied as five spots approx. 2 mm. apart. All acetone-insoluble components were detected with (a) I₂ vapour followed by spraying with a 1% soluble-starch solution, or (b) Rhodamine G6 (Dittmer & Lester, 1964). Amino lipids were detected with 0.25% ninhydrin in acetone-lutidine-acetic acid (50:2:15, by vol.), choline lipids with Dragendorff's reagent (Doizaki & Zieve, 1963), aldehyde groups with the Schiff-Feulgen reagent (Skidmore & Entenman, 1962), vicinal hydroxyl groups with periodate-Schiff reagent (Buchanan, Dekker & Long, 1950), phospholipids with HClO₄-molybdate reagent (Skipski, Peterson & Barclay, 1962), sulpholipids with NaN₃-I₂ (Huston, Albro & Grindley, 1965), and phosphatidic acid and phosphatidyl-glycerol with ammoniacal AgNO₃ (Huston *et al.* 1965); ³²P- and ³⁵S-labelled lipids were detected by radioautography on Ilford X-ray film. Purified egg-yolk phospholipids were chromatographed for comparative R_F values. Carotenoid pigments were detected visually, by spraying with Rhodamine G6, and by their ultraviolet absorption. The two most concentrated red pigments were eluted from the plate with the solvent used for chromatography and re-run on silicic acid plates until pure. The visible-absorption spectra of these compounds in chloroform were recorded. Amino acids were chromatographed with the mixed-layer adsorbent and solvent systems described by Turner & Redgwell (1966). The water-soluble phosphate esters and other products of acid hydrolysis (see below) were chromatographed on Whatman no. 1 paper in butanol-acetic acid-water (6:1:2, by vol.) and phenol-water (4:1, w/v).

Lipid P was determined by the modified Bartlett procedure described by Marinetti (1962). Lipid N was

estimated by Nessler's method (King & Wootton, 1956) and by that of Sloane-Stanley (1967). Amino N was measured by the method of Troll & Cannan (1953) and Lea & Rhodes (1955) with glycine as standard. Yields of lipid fractions were determined gravimetrically. Separated acetone-insoluble components were removed from thin-layer plates by scraping the adsorbent from the plate and eluting it three times with 3 ml. of the solvent used for chromatography. The solvent was evaporated to dryness and P was estimated.

Water-soluble phosphate esters were obtained by hydrolysing a sample of acetone-insoluble lipids with 6 N-HCl for 2-5 hr. at 100° (Sehgal *et al.* 1962). Mild alkaline hydrolysis was by the procedure of Dawson (1954). Strong acid hydrolysis consisted of heating samples of lipid (1-2 mg.) with redistilled constant-boiling HCl (1.0 ml.) in a sealed tube at 105° overnight. The hydrolysate was cooled in ice and the fatty acids were removed by extracting it three times with diethyl ether (2 ml.). The pooled ether extracts were washed once with water (2 ml.). The aqueous fraction was repeatedly evaporated on a steam bath under N₂ to remove HCl, and made up to a convenient volume with water before use.

The AgNO₃-NaOH and aniline phthalate spray reagents were used for the detection of carbohydrates; the phosphomolybdic acid-SnCl₂ reagent was used to detect choline (Block, Durrum & Zweig, 1958). [³²P]Phosphate esters were located on chromatograms with a Nuclear-Chicago Actigraph II model 1036 Chromoscan. The amino acid composition of the acid-hydrolysed acetone-insoluble lipid was determined conventionally with a Beckman model 120C amino acid analyser. Infrared-absorption spectra of solutions of lipids in CCl₄ were obtained with a Perkin-Elmer model 221 infrared spectrophotometer. Visible and ultraviolet absorptions were measured on a Unicam SP.800 recording spectrophotometer.

RESULTS

Growth of the organism and membrane yield. The mean generation time was about 13 hr.; exponential growth ceased after about 5 days. The yield of membrane/mass of organisms was determined in arbitrary units by dividing the mass of isolated membranes by the turbidity ($E_{680}^{1\text{cm.}}$) of the culture from which they were obtained (Fig. 1). There was a somewhat increased yield of membrane at the end of exponential growth.

Content of lipid. The membrane lipid content increased from 15% (w/w) after 3 days to about 21% after 5 days growth and decreased to 17% after 8-9 days.

Lipid nitrogen. The range of total nitrogen content of the lipid was about 6-10 µg. of nitrogen/mg. of lipid. The maximum was obtained at 4-5 days of growth, after which the content decreased again to about 6 µg./mg.

Phospholipids. The gross composition of the lipid changed during the growth cycle, with an increase in the proportion of acetone-insoluble lipids up to the end of exponential growth (Table 1).

The phosphorus content of total membrane lipids increased from about 18 $\mu\text{g.}/\text{mg.}$ of lipid early in exponential growth to about 40 $\mu\text{g.}/\text{mg.}$ of lipid at the end of exponential growth (Table 2). The phosphatide content of the total lipid almost doubled between 1.5 and 3 days of culture. After 4 days, however, there was no appreciable change in the phosphatide portion of the acetone-insoluble lipids. Table 3 lists the phosphorus and nitrogen composition of the acetone-insoluble fraction from stationary-phase (6-day) cultures. Chromatography of the lipids on thin-layer plates revealed 12

Fig. 1. Effect of the age of the culture on the yield of membrane. Yield is expressed in arbitrary units calculated as described in the text. Δ , $\times$ and $\circ$, Three different experiments.

Table 1. Effect of age of culture on gross lipid composition

Age of culture (days)	Acetone-insoluble lipids (%)		Acetone-soluble lipids (%)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
3	63	59	37	41
4	59	69	42	31
5	67	70	34	30
6	63		36	
7	74		26	
8		74		26
9		74		26

components in the acetone-insoluble fraction. One component that chromatographed very close to the origin was absent from lipids extracted from dialysed lipoproteins. Although this component stained with iodine-starch it did not stain with Rhodamine G6 and was probably a non-lipid contaminant. A tentative identification of the acetone-insoluble components by means of various spray reagents is shown in Table 4.

Mild alkaline hydrolysis of the acetone-insoluble lipids did not release phosphate esters. Short acid hydrolysis (2.5 hr. in 6N-hydrochloric acid at 100°) released five major and two minor phosphate-positive components (i.e. components that produced a blue colour with the molybdate-perchloric acid spray reagent), six of which could be measured as $[^{32}\text{P}]$ phosphate esters with the Chromoscan (Table 5). Glycerol 1-phosphate and glycerol 1,3-diphosphate were released in the approximate molar ratio 2:1 (i.e. equal amounts of phosphorus). No glycerylphosphorylglycerol was detected. Neither glycerylphosphorylethanolamine nor inositol monophosphate was demonstrated after mild alkaline hydrolysis. Glycerylphosphorylcholine was not detected after mild alkaline hydrolysis, though strong acid hydrolysis of the lipid released traces of choline. The other phosphate compounds were not identified.

Acid hydrolysis (16 hr. in 6N-hydrochloric acid at 105°) of compound 4 (Table 4) yielded mannose, galactose and glucose. The ultraviolet-absorption spectrum of the acetone-insoluble lipids showed a

Table 3. Phosphorus and nitrogen composition of acetone-insoluble lipids extracted from the membrane of *H. halobium*

Component	Content in acetone-insoluble lipid fraction (%)
P	4.3
N	0.6-0.8
Amino N from unhydrolysed lipid	0.0075
Amino N after acid hydrolysis	0.3-0.47
N/P atomic ratio	0.31-0.41

Table 2. Effect of age of culture on the total phosphorus content of the total lipid

Expt. 1		Expt. 2		Expt. 3	
Age of culture (days)	% P	Age of culture (days)	% P	Age of culture (days)	% P
1.5	1.78	3	3.30	3	3.26
2.5	2.55	4	3.04	4	3.72
3.5	2.81	5	4.45	5	4.03
5.5	3.59	6	4.20	8	3.87
8.5	3.60	7	4.18	9	3.89

Table 4. *Reactions and tentative identification of the acetone-soluble lipids*

R_F values were obtained on thin-layer chromatograms developed with chloroform-methanol-water (65:25:4, by vol.). The Rhodamine 6G test results are indicated Y, yellow; B, blue; and G, grey, when viewed under ultraviolet light. Periodate-Schiff reactions appeared to be rapid. Spots 2, 9 and 10 were present in about twice the proportion in extracts from undialysed wet membrane preparations as in extracts of dialysed freeze-dried lipoproteins.

Spot no.	R_F	I ₂ -starch	Rhod-amine G6	Phosphate and ³² P	Dragendorff	Periodate-Schiff	Schiff-Feulgen	Ninhydrin	AgNO ₃ -aq. NH ₃ soln.	Azide-I ₂	³⁵ S	Tentative identification	Proportion of total P present in spot (%)
1	0.03	+	-	-	-	-	-	-	-	-	-	Non-lipid contamination	0
2	0.10	+	Y	+	-	-	-	+	-	-	-	Amino acid ester of phosphatidylglycerol (unidentified)	Not determined
3	0.20	+	Y	+	+	-	-	-	-	-	-	Lecithin (ether form)	Not determined
4	0.26	+	Y	-	-	+	-	-	-	+	+	Glycolipid sulphate ester	0
5	0.37	+	Y	+	-	+	-	-	+	-	-	Unidentified	Not determined
6	0.39	+	B	+	-	+	-	-	-	+	-	Unidentified	Not determined
7	0.51	+	B	-	-	+	+	Weak	-	+	+	Lipopeptide	0
8	0.59	+	Y	+	-	+	+	-	-	-	-	Unidentified	2.5 ± 0.5
9	0.70	+	B	-	-	+	-	-	+	-	-	Glycolipid	0
10	0.81	+	Y	+	-	+	+	-	+	-	-	Phosphatidylglycerol (diether form)	7.5 ± 0.7
11	0.82 to 0.92	+	B/G	+	-	-	-	-	-	-	-	Phosphatidylglycerophosphate (diether form)	78 ± 3
12	0.95	+	B	+	-	-	-	-	+	-	-	Phosphatidic acid (diether form)	0.4 ± 0.1
13	0.99	+	Y	-	-	-	-	-	-	-	-	Traces of pigment	Not determined

Table 5. *Relative amounts of ^{32}P in phosphate esters released by acid hydrolysis of acetone-insoluble lipids*

The ^{32}P proportion is the area under the curve drawn by the Actigraph chromoscan recorder, expressed as a percentage of the area for spot 6. Spots 2 and 6 were glycerol 1,3-diphosphate and glycerol 1-phosphate respectively; the identity of the other phosphate esters was not established with certainty.

Spot no.	R_F in butanol-acetic acid-water (6:1:2, by vol.)	^{32}P proportion (%)
1	0	92
2	0.024	98
3	0.080	45
4	0.104	18
5	0.139	53
6	0.420	100

Table 6. *Amino acid composition of the total acetone-insoluble lipids*

	Amino acid composition (moles/100 moles)
Ala	13.84
Arg	8.77
Asp	9.33
Cysteic acid	2.13
Glu	6.08
Gly	10.64
His	5.88
Ile	4.05
Leu	9.43
Lys	5.07
Phe	3.14
'Serine phosphate'	1.27
Pro	6.69
Ser	5.73
Thr	6.79
Tyr	1.22

maximum at $280\text{m}\mu$ and several amino acids were released by strong acid hydrolysis of this material. The hydrolysate also contained an amino acid tentatively identified as serine phosphate (Table 6). This amino acid mixture was released from lipid that had been subjected to the purification described above.

Infrared-absorption spectra of acetone-insoluble lipids were similar to those obtained by Kates, Palameta, Joo, Kushner & Gibbons (1966) for lipids extracted from whole cells of two strains of *H. halobium* and to that of the pure disodium salt of the diether analogue of phosphatidylglycerophosphate (Kates, Yengoyan & Sastry, 1965). The absence of fatty acid esters has already been reported (Sehgal *et al.* 1962; Cho & Salton, 1966), and this was confirmed by the lack of fatty ester

Fig. 2. Thin-layer chromatogram of acetone-insoluble lipids ($20\text{ }\mu\text{g. of P}$) separated in chloroform-methanol-water (65:25:4, by vol.). The spots were detected by exposing the chromatogram to I_2 vapour and then spraying it with a 1% (w/v) solution of soluble starch. The samples (from left to right) are from 3-, 4-, 5-, 8- and 9-day cultures. The numbered bands correlate with Table 4. Spot 2 is the ninhydrin-positive compound that is present only in young cultures. Spots 5 and 6 are phospholipids that reach maximal concentration at the end of exponential growth (see the text). Spot 7, which has been tentatively identified as a lipopeptide, decreases slightly after 3 days. The proportion of phosphatidylglycerol (spot 10) also decreases appreciably after 3 days' culture.

absorption between 1735 and 1750cm.^{-1} in these preparations. The characteristic absorption caused by methyl branching of the dihydrophytyl side chains (1375 – 1385cm.^{-1}) was also evident in both acetone-soluble and acetone-insoluble fractions. Ethers absorbed at 1095cm.^{-1} and P–O–C bonds at 1055cm.^{-1} . The absorption of the acetone-insoluble lipids at 1645cm.^{-1} could be indicative of the amide I C=O bond, though amide II absorption at 1540cm.^{-1} was only slight. Feeble absorption of the neutral-lipid fraction at 1740cm.^{-1} and 1710cm.^{-1} confirmed the low content of glycerides and free fatty acids respectively. Quinones were not detected by infrared absorption or thin-layer chromatography.

Variations in the composition of the acetone-insoluble fraction were detected chromatographically and are illustrated in Fig. 2. A conspicuous change that occurred in late exponential growth

Fig. 3. Thin-layer chromatography of the acetone-soluble extract from the membrane of *H. halobium*. The solvent system was light petroleum (b.p. 40–60°)–acetone–diethyl ether–acetic acid (70:20:10:1, by vol.). Non-pigmented spots were detected with I_2 vapour or Rhodamine G6. The pigments all absorbed ultraviolet light. The colours of the pigments are as follows: 1, orange; 2, red; 3, orange; 4, yellow; 5, mauve; 6, red; 7, orange-red; 8, yellow; 9, yellow; 10, red; 11, red; 12, orange; 13, yellow; 14, orange; 15, yellow; 16 and 17, colourless.

was a decrease in the proportion of a ninhydrin-positive component. Phosphatidylglycerol (diether form) appeared to decrease appreciably after 3 days' growth. Two unidentified components (spots 5 and 6), which contained phosphorus and which were oxidized by periodate, increased to a maximum in mid-stationary phase. After 8 days these compounds were present only in traces. No changes in the proportion of the major phospholipid, phosphatidylglycerophosphate (diether form) (spot 11), were apparent from inspection of the chromatograms.

Acetone-soluble fraction. Thin-layer chromatography of the acetone-soluble lipid fraction revealed 15 pigments, four of which were present in low proportions. Two unidentified colourless components were also present in this fraction. Fig. 3 illustrates the resolution of the pigment fraction

on a thin-layer chromatogram. The visible spectrum of a major red band (spot 6) had absorption peaks at 463, 493 and 523m μ . Spot 7 had absorption peaks at 454, 482 and 505m μ . Component 6 was present in slightly higher proportion than component 7. The proportion of carotenoid pigments in the membrane increased with the age of the culture. Fig. 4 shows the extinction of solutions of lipid in acetone and of lipoprotein in buffered urea (approx. 8M-urea, pH 8.9; the cathode buffer for gel electrophoresis; A. D. Brown & R. F. Pearce, unpublished work). Both methods showed that the proportion of material absorbing at 500m μ was greatest at the end of exponential growth.

DISCUSSION

The increase in yield of membrane from about 100 units in late exponential cultures to 130 units in the stationary phase was consistent with a decrease in cell size, the experimental error being reflected in Fig. 1. The gross change in membrane composition during growth was equivalent to an increase of 40% in the lipid content. This value is similar to the change in composition of the protoplast membrane of *Streptococcus faecalis* between exponential- and stationary-growth phases in a medium limited by valine (Shockman, Kolb, Bakay, Conover & Toennies, 1963); the lipid content of the *S. faecalis* membrane was 28–40%.

A lipid content of about 20% is similar to that of the envelope of *H. cutirubrum* (Kushner, Bayley, Boring, Kates & Gibbons, 1964) and the inner membranes of ox heart and rat liver mitochondria (Parsons, 1966). The lipid mixture, of which 80% is insoluble in acetone, is highly polar. More specifically it is highly acidic, since 80% of the membrane lipid phosphorus occurs in the diether analogue of phosphatidylglycerophosphate and the proportion of basic substituents is very low. Phosphatidylglycerophosphate accounts for 73% of the lipid phosphorus in *H. cutirubrum*, which also lacks appreciable quantities of nitrogenous lipids (Sehgal *et al.* 1962). Although the characteristic disaggregation of the membrane of *H. halobium* is caused directly by $-CO_2^-$ groups on the membrane proteins (Brown, 1963, 1965), the highly acidic membrane lipids must necessarily contribute to the halophilic properties by neutralizing basic groups on the protein and contributing to the orientation of the proteins on the membrane surface (cf. Brown, 1965). Moreover, the lipid becomes more acidic with increasing age of the culture (Table 2).

The method of extracting lipid from freeze-dried lipoproteins used as a routine in this work gave the same polar lipids as extracting wet preparations by the method of Kates and his co-workers (Sehgal *et al.* 1962; Kates *et al.* 1966). The only apparent

Fig. 4. Effect of the age of the culture on the content of carotenoid pigments in the membrane. (a) Extinction at $500\text{m}\mu$ of the total acetone-soluble lipid fraction in acetone (1 ml.), divided by the turbidity of the culture (E_{880}) from which it was obtained. This is the unit of concentration used by Baxter (1960). (b) Extinction at $500\text{m}\mu$ of a solution of membrane lipoprotein (4 mg./ml.) in buffered urea, pH 8.9 (the cathode buffer for gel electrophoresis of the lipoproteins; A. D. Brown & R. F. Pearce, unpublished work).

differences between the two methods were the absence of a non-lipid component (spot 1, Table 4) from the dry extract, and different proportions of some components. Such changes in proportion might be expected to arise from incomplete extraction or enzyme action (e.g. during dialysis). Extraction of lipid from dialysed lipoproteins was considered to be more relevant to other phases of the investigation.

Phosphatidylglycerol (ether analogue), which contained about 7.5% of the membrane lipid phosphorus, has been reported in whole-cell lipids of other strains of *H. halobium* (Kates *et al.* 1966), as has the glycolipid sulphate ester (Kates *et al.* 1966; Kates, Palameta, Perry & Adams, 1967). Of the seven spots that gave rapid reactions with the periodate-Schiff reagent (Table 4), three were unidentified compounds (spots 5, 6 and 8) not previously reported from halophilic bacteria, one was a presumed glycolipid (spot 9) and one (spot 7) contained a bound peptide (see below). The glycolipid was one of the compounds extracted in lower proportion from freeze-dried lipoproteins than from wet membrane preparations.

Three nitrogenous compounds were detected in the polar-lipid fraction. Two of these appeared to be phospholipids and the third, mentioned above, contained a bound peptide. Lecithin (spot 3) was present in quantities too low to permit valid phosphorus estimations on the eluted material. Failure to detect glycylphosphorylcholine after mild alkaline hydrolysis was consistent with the evidence already cited that lecithin, like the other phospholipids, occurred as the diether analogue. Spot 2, which reacted with ninhydrin, was extracted

in higher proportion from the membranes of organisms in the early exponential growth phase and from undialysed membrane preparations. This compound had the properties of a 'lipoamino acid' (cf. Macfarlane, 1962, 1964), which is likely to be sensitive to hydrolysis, including possible enzymic hydrolysis during dialysis. Spot 7 reacted weakly with ninhydrin and formed a hydroxamate, suggesting an esterified carboxyl group. Hydrolysis of rigorously purified acetone-insoluble lipid (see the Materials and Methods section) released about 16 amino acids, with an excess of the basic ones (Table 6), and caused a 50-fold increase in amino nitrogen (Table 3). One amino end group was detected. The basicity of the peptide distinguished it from the membrane proteins; its amino acid composition and other properties were different from those of a 'proteolipid' extracted from the membrane of *H. halobium* (McClare, 1967). Lipopeptides have been obtained from hen oviducts (Hendler, 1963a,b) and rat kidney (Schwartzman, Crawhall & Segal, 1966), but to our knowledge, not from Gram-negative bacteria. The presence of this compound was probably partly responsible for the relatively high nitrogen/phosphorus atomic ratio of the acetone-insoluble lipid fraction (about 0.4 compared with 0.09 for the corresponding fraction from *H. cutirubrum*; Sehgal *et al.* 1962).

The acetone-soluble fraction was also more complex than reported previously for this or related organisms. For example, Baxter (1960) separated eight pigments from *H. halobium*, the major one being α -bacteriorubrin. Both α - and β -bacteriorubrin occurred as major components in

the present preparations. The results summarized in Fig. 3 agree with Baxter's (1960) findings of an increase in carotenoid pigmentation with increasing culture age of *H. salinarium*. Table 1 and Fig. 4 together show that the carotenoid content of the acetone-soluble lipid fractions increased with age of culture.

We thank Mr J. B. Davenport of the Division of Food Preservation, Commonwealth Scientific and Industrial Research Organisation, Ryde, Sydney, for valuable advice throughout this work and Mr A. Scott, of the same address, for the infrared-absorption measurements. We are grateful to Mr R. W. Hosken of the Department of Biochemistry, University of New South Wales for the amino acid analysis.

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