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The Effects of Temperature on the Composition and Physical Properties of the Lipids of *Pseudomonas fluorescens*

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1. *Pseudomonas fluorescens* was grown at various temperatures between 5°C and 33°C. The extractable lipids from organisms at various stages of growth and grown at different temperatures were examined. 2. The extractable lipids contained phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, and an ornithine-containing lipid. The relative amounts of these lipids did not vary significantly during growth or with the changes in growth temperature. 3. The major fatty acids were hexadecanoic, hexadecenoic and octadecenoic acids and the cyclopropane acids methylene-hexadecanoic and methylene-octadecanoic acids. The relative amount of unsaturated acids (including cyclopropane acids) did not change significantly during growth, but increased with decreasing temperature. 4. Phosphatidylethanolamines with different degrees of unsaturation and containing different amounts of cyclopropane acids were isolated from organisms grown at 5°C and 22°C and their surface and phase behaviour in water was investigated. Thermodynamic parameters for fusion and monolayer results for cyclopropane and other fatty acids were examined. 5. The surface pressure–area isotherms of phosphatidylethanolamines containing different amounts of unsaturated fatty acids show small differences but the individual isotherms remain essentially unchanged over the temperature range 5–22°C. X-ray-diffraction methods show that the structures (lamellar+hexagonal) formed in water by phosphatidylethanolamine, isolated from organisms grown at 5°C and 22°C, are identical when compared at the respective growth temperatures. This points to a control mechanism of the physical state of the lipids that is sensitive to the operating temperature of the organism. 6. The molecular packing of cyclopropane acids is intermediate between that of the corresponding *cis*- and *trans*-monoenoic acids. However, substitution of a cyclopropane acid for a *cis*-unsaturated acid has insignificant effects on the molecular packing of phospholipids containing these acids.

The lipid compositions of a variety of biological systems show an increase in unsaturation with a decrease in environmental temperature. The reasons for this phenomenon are not well understood and various hypotheses have been advanced. The physical properties of fatty acid chains in phospholipids play a critical role in any theory that suggests that the fatty acid composition is dictated by functional requirements of the permeability barrier of an organism. There have been few systematic physical studies to support such claims.

Similar effects of growth temperature have been observed in some bacterial lipids, where, however, the fatty acid composition may vary with medium constituents, pH, aeration and the phase of growth (O'Leary, 1967). In bacteria, cyclopropane acids are synthesized from monounsaturated acids esterified in phospholipid molecules (Zalkin, Law & Goldfine, 1963; Thomas & Law, 1966) and reports of the degrees of unsaturation are usually presented with the cyclopropane acids included in the unsaturated fatty acid content. However, before such results can be employed to support any physical theories it is necessary to demonstrate that the physical properties of cyclopropane and monounsaturated fatty acids are equivalent.

This paper describes the changes in the extractable lipids of *Pseudomonas fluorescens*, a cold-tolerant organism, during the growth cycle at various temperatures in the range 5–33°C. *P. fluorescens* was grown at two temperatures and the monolayer packing and bulk-phase behaviour

of the phosphatidylethanolamine extracted from the organisms was measured at the same two temperatures. The thermodynamic parameters for fusion and monolayer results for a selection of relevant fatty acids were compared.

METHODS

Growth of organisms. The organism was *Pseudomonas fluorescens* N.C.M.B. 129. Organisms were grown in 2-litre Erlenmeyer flasks each containing 200ml of nutrient broth (Oxoid CM1, Oxoid Ltd., London E.C.4, U.K.). Flasks were shaken on a rotary shaker (Model V, New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) in a constant-temperature room at the stated temperatures, and the air temperature immediately above the shaking platform was continuously monitored. At the times stated cells were harvested by centrifuging, washed once with 0.85% NaCl and either freeze-dried or stored below −10°C. Each inoculum was prepared by transferring cells from a slope of Nutrient Agar (Oxoid CM3) grown at 22°C to 15ml of nutrient broth in a 100ml Erlenmeyer flask that was shaken at the required temperature (8h at 33°C, 16h at 30°C and 22°C, 24h at 15°C, 48h at 9°C, 74h at 5°C). Inocula (1ml) were diluted before use with 9ml of Ringer solution (Oxoid BR52); 0.05ml of diluted inoculum was added to each 2-litre Erlenmeyer flask containing 200ml of nutrient broth. Nutrient broth and Ringer solution were stored at the growth temperature before use.

Two 1ml samples of culture liquor were removed at regular intervals for duplicate measurement of cell density. Not more than 20ml (usually 12–16ml) was removed from any one flask during an experiment. Cell density was measured against a water blank by using an EEL Absorptiometer (Evans Electroselenium Ltd., Halstead, Essex, U.K.) fitted with a neutral-density filter. Cell density was correlated with dry weight concentration of cells during exponential and post-exponential growth.

Extraction and chromatography of lipids. Freeze-dried or wet cells were suspended in methanol by means of a tissue grinder and the suspension was stirred at 60°C for 15min. The suspension was centrifuged and the insoluble material was re-extracted with methanol as before, then with four portions of chloroform at room temperature. The extracts were combined, concentrated to dryness, dissolved in chloroform and filtered. The chloroform solution was concentrated to dryness and dried *in vacuo*. The crude lipid was freed from non-lipid components by the treatment of Folch, Lees & Sloane-Stanley (1957). Lipids were examined by t.l.c. on silica gel G plates (Merck A.-G., Darmstadt, Germany) with chloroform–methanol–water (65:25:4, by vol.) (Wagner, Hörhammer & Wolff, 1961) and the two-dimensional system chloroform–methanol–7M-NH_3 (65:30:4, by vol.) and chloroform–methanol–acetic acid–water (170:24:25:4, by vol.) (Nichols, 1964). Lipids were detected by using ninhydrin solution (0.5%, w/v, in butan-1-ol), chromic acid and Zinzadze's reagent (Dittmer & Lester, 1964). Lipids were fractionated by chromatography on columns of DEAE-cellulose (acetate form) (Whatman ion-exchange cellulose DE23, W. & R. Balston Ltd., Maidstone, Kent, U.K.) as described by Rouser, Kritchevsky, Heller & Lieber (1963).

Methyl esters were prepared by interesterification of lipid with boron trifluoride–methanol reagent (approx. 14% BF_3, BDH Chemicals Ltd., Poole, Dorset, U.K., laboratory reagent) at 60°C for 30min. Methyl esters were extracted with light petroleum (b.p. 30–40°C) after addition of water and examined by g.l.c. with a Perkin–Elmer F11 gas chromatograph with a dual flame-ionization detector and columns of 20% (w/w) poly-(diethyleneglycol succinate) on 'C' 85–100 BSS mesh diatomite support (JJ's Chromatography Ltd., Kings Lynn, Norfolk, U.K.). Peak areas were measured by the method of Carroll (1961).

Deacylation of lipids. Lipid extracts were hydrolysed under mild alkaline conditions (Ballou, Vilkas & Lederer, 1963). The water-soluble products were examined by paper chromatography (Whatman no. 1 paper) with phenol–water (100:38, w/v) and butan-1-ol–propionic acid–water (142:71:100, by vol.) (Benson & Strickland, 1960) and stained with the reagent of Hanes & Isherwood prepared as described by Bandurski & Axelrod (1951). Glycerophosphorylethanolamine and glycerophosphorylglycerol were identified by comparison with authentic compounds prepared from pure lipids; 1,3-diglycerophosphorylglycerol was identified by comparison with the results of Benson & Strickland (1960).

The chloroform-soluble products of the mild alkaline hydrolysis were examined by t.l.c. The chloroform solution was concentrated to dryness and hydrolysed in 6M-HCl at 100°C for 4h. The acid hydrolysate was concentrated to dryness, suspended in water and extracted with diethyl ether. The aqueous residue after extraction was examined by paper chromatography (Whatman no. 1 paper) with butan-1-ol–acetic acid–water (67:10:23, by vol.) and methanol–water–pyridine–HCl (32:7:4:1, by vol.) and stained with the polychromatic ninhydrin stain for amino acids (Moffat & Lytle, 1959).

Physical studies of lipids. Phosphatidylethanolamine (with a trace amount of ornithine-containing lipid) isolated from crude lipid of *P. fluorescens* was precipitated from benzene solution with acetone and freeze-dried from benzene solution. The fatty acids for which the melting points are listed in Table 4 were all chromatographically pure samples. The oleic, elaidic and dihydrosterculic acid samples were prepared in this laboratory. The oleic acid, which had been zone-refined, and the dihydrosterculic acid, which was synthetic and therefore racemic, were generous gifts from Dr N. Albon and Dr L. J. Morris respectively. The petroselinic acid and *cis*-vaccenic acid were purchased from the Sigma (London) Chemical Co. (London S.W.6, U.K.).

The apparatus and techniques for measuring surface pressure as a function of molecular area for lipids have been described by Phillips & Chapman (1968). The molecular areas were reproducible to ± 1.5 Å^2/molecule and the surface pressures to ± 0.75 dyn/cm. Homogeneous dispersions of the phosphatidylethanolamine from *P. fluorescens* were prepared for X-ray diffraction measurements by repeated centrifugation of mixtures of lipid and water through a narrow constriction in a glass capillary. Phosphatidylethanolamine–water samples were then sealed in a variable-temperature holder and their low-angle X-ray-diffraction patterns recorded as a function of temperature (0°C–80°C). A Rigaku–Denki camera using Cu K_α radiation and a sample-to-detector distance

of 200mm gave diffraction patterns in the range $1/200 < 1/d < 1/15$ (Å^{-1}), where d is the lamellar repeat or distance between rows of cylinders. The melting properties of the fatty acids were examined by using a Perkin–Elmer DSC-1 differential scanning calorimeter. The techniques have been thoroughly described by Ladbrooke & Chapman (1969).

RESULTS

P. fluorescens was grown at 33°C, 30°C, 22°C, 15°C, 9°C and 5°C; the organism failed to grow above 34°C. The cell yield, measured as cell density, was not affected by the growth temperature. An Arrhenius plot of the specific growth rate of *P. fluorescens* indicated an optimum temperature of about 30°C and the linear portion of the curve extended from 30° to 9°C. The temperature characteristic of growth (μ), a value corresponding to the activation energy of a chemical reaction, calculated from the linear portion of this curve, was 11060cal/mol. This is similar to the value of 10700cal/mol calculated from the results of Ingraham (1958) for *P. fluorescens* (strain P-200, WRRL) between 32°C and 14°C.

Effect of temperature on the composition of the extractable lipids. The composition of the extractable lipids was examined at intervals during the growth cycle. Since the stationary phase of growth varied in duration with temperature, incubation was continued into the phase of decline, manifested here by a decrease in observed cell density. No correlation between cell-density measurements and cell concentration was sought for cultures in these later phases of the growth cycle. At each of the six growth temperatures, the first sample of cells was harvested during the exponential phase of growth, the second sample as near to the end of the exponential phase as possible and subsequent samples were harvested during the post-exponential and stationary phases of growth and the phase of decline. Lipids were extracted from all samples and examined by t.l.c. Selected samples of cells were freeze-dried before extraction and the extractable lipid, freed from non-lipid components, was approx. 6–7% of the dry weight of the cells; this amount did not vary significantly with growth temperature. Crude lipids were fractionated from cells grown at 30°C, 22°C and 5°C. Table 1 shows that the phospholipids were 80–90% of the washed lipid, the remainder being pigments and small amounts of neutral lipids. The nature of the pigments, the completeness of their extraction and variation with growth temperature were not investigated. Phosphatidylethanolamine constituted approx. 75% of the phospholipids. Separation of the two acidic phospholipids by t.l.c. yielded a diphosphatidylglycerol/phosphatidylglycerol ratio of 2:1 (w/w) giving an overall approximate composition of 75% phosphatidylethanolamine, 16% diphosphatidylglycerol and 8% phosphatidylglycerol and a small amount of phosphatidylcholine. The lipid composition, as detected by t.l.c., did not apparently change during the growth cycle and the relative proportions of the phospholipids did not change significantly with growth temperature (Table 1). Phospholipids were identified by their behaviour and staining reactions on t.l.c. and the identities of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol were confirmed by examination of the water-soluble products of deacylation.

A trace amount of an unknown component that contained ornithine but lacked phosphorus was present in the extractable lipid. This component was also detected in the chloroform-soluble products of deacylation of lipid extracts, where a single ninhydrin-positive component corresponding to ornithine was detected after acid hydrolysis. This ornithine-containing lipid was eluted from DEAE-cellulose (acetate) together with phosphatidylethanolamine and was detected by t.l.c. in chloroform–methanol–water or chloroform–methanol–

Table 1. *Composition of the extractable lipid of* P. fluorescens

Temperature ...	30°C		22°C		22°C		5°C	
Phase of growth ...	Stationary phase		Post-exponential phase		Stationary phase		Stationary phase	
Components	Weight (%)	% of phospholipids	Weight (%)	% of phospholipids	Weight (%)	% of phospholipids	Weight (%)	% of phospholipids
Pigments, Neutral lipids	14.9		15.3		9.2		14.7	
Phosphatidylcholine					1.8	2		
Phosphatidylethanolamine, Ornithine-containing lipid	63.9	75	62.8	74	67.4	74	65.8	77
Phosphatidylglycerol, Diphosphatidylglycerol	21.2	25	21.9	26	21.5	24	19.4	23

ammonia where it moved just behind phosphatidylethanolamine. The ornithine-containing lipid was detected in trace amounts in the extractable lipid from cells at all stages of growth at each growth temperature examined and showed some resemblance to the ornithine-containing lipids found in certain other organisms, for example *Rhodopseudomonas spheroides* (Gorchein, 1964, 1968).

The total fatty acids of the extractable lipid from all samples of cells were examined by g.l.c. The fatty acids were $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0\Delta}$, $C_{18:0}$, $C_{18:1}$ and $C_{19:0\Delta}$ with trace amounts of $C_{12:0}$ and components with carbon numbers [determined on columns of poly-(diethyleneglycol succinate)] 15.0, 17.0 and 19.0 (Δ indicates the presence of a cyclopropane ring). At all temperatures exponentially growing cells contained less than 1.0% (expressed as mol/100 mol of fatty acids) of $C_{17:0\Delta}$ and no $C_{19:0\Delta}$. After the end of the exponential phase of growth there was accumulation of $C_{17:0\Delta}$, and to a lesser extent $C_{19:0\Delta}$, at the expense of the corresponding unsaturated acid. The effect of decreasing the growth temperature was to decrease this accumulation of cyclopropane acids. The changes in fatty acid composition during growth at 30°C and at 5°C are shown in Figs. 1 and 2 respectively. Since cyclopropane acid is formed from a monounsaturated acid esterified in a phospholipid (Zalkin *et al.* 1963; Thomas & Law, 1966) the total degree of unsaturation of the phospholipids is reflected in the sum of cyclopropane acids and

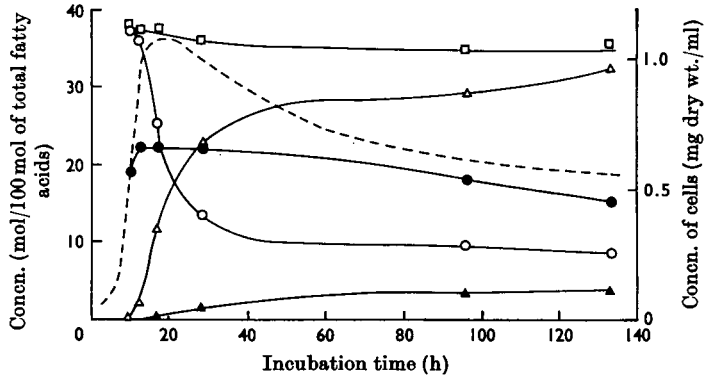

Fig. 1. Changes in the fatty acid composition of the extractable lipid of *P. fluorescens* during growth at 30°C. Values are given as mol/100 mol of total fatty acids. □, $C_{16:0}$; ○, $C_{16:1}$; ●, $C_{18:1}$; △, $C_{17:0\Delta}$; ▲, $C_{19:0\Delta}$. The concentration of cells (mg dry wt. of cells/ml) (-----) is approximate for the later stages of the growth cycle.

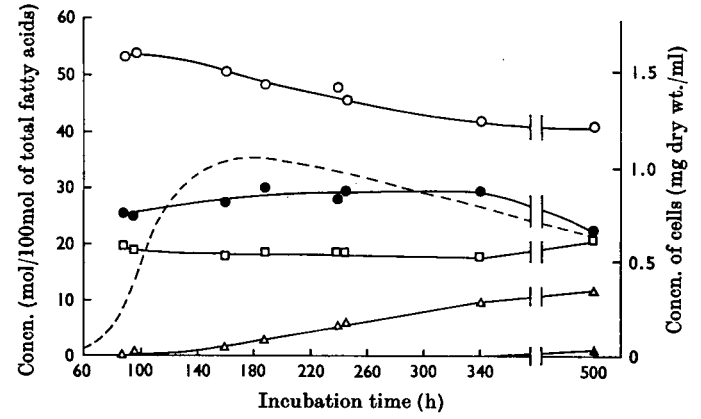

Fig. 2. Changes in the fatty acid composition of the extractable lipid of *P. fluorescens* during growth at 5°C. □, $C_{16:0}$; ○, $C_{16:1}$; ●, $C_{18:1}$; △, $C_{17:0\Delta}$; ▲, $C_{19:0\Delta}$. The concentration of cells (mg dry wt. of cells/ml) (-----) is approximate for the later stages of the growth cycle.

Table 2. *Effect of the growth temperature on the fatty acid composition of the extractable lipid of* P. fluorescens

The number of samples of cells harvested during growth at each temperature is indicated. The phases of growth at which samples were taken are described in the text. The mean and S.D. for the total unsaturated acids and the major components, expressed as mol per cent of fatty acids, are given for each temperature. Minor components, $C_{14:0}$, $C_{18:0}$ and $C_{20:0}$, are not included in the table. △ indicates the presence of a cyclopropane ring.

	Composition (mol/100 mol of fatty acids)											
Temperature ...	33°C		30°C		22°C		15°C		9°C		5°C	
No. of samples ...	4		6		8		7		10		8	
Fatty acid	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
$C_{16:0}$	37.8	3.0	36.3	1.2	27.5	1.5	22.7	1.5	20.3	1.3	18.7	0.9
$C_{16:1}+C_{17:0\Delta}$	33.2	1.6	38.0	1.4	44.6	1.4	47.2	1.1	48.6	2.7	52.4	1.0
$C_{18:1}+C_{19:0\Delta}$	25.6	1.4	21.3	1.7	25.3	1.5	28.9	1.5	29.2	2.1	25.2	2.3
Unsaturated acids	58.9	2.4	59.3	1.3	69.6	1.2	76.1	1.5	77.8	2.0	79.6	1.7

Table 3. *Fatty acid compositions of the extractable lipid and phosphatidylethanolamine of* P. fluorescens *grown at* 22°C *and* 5°C

Phosphatidylethanolamine was isolated from lipid extracts of cells grown at: (i) 22°C for 20h (post-exponential phase of growth), (ii) 22°C for 48h (stationary phase) and (iii) 5°C for 165h (stationary phase).

	Composition (mol/100 mol of fatty acids)					
	Extractable lipid		Phosphatidyl-ethanolamine		Extractable lipid	Phosphatidyl-ethanolamine
Fatty acid	22°C/20h	22°C/48h	22°C/20h	22°C/48h	5°C/165h	5°C/165h
$C_{14:0}$	0.6	1.0	0.2	0.2	0.2	2.3
$C_{16:0}$	30.7	26.5	34.7	32.2	20.0	21.2
$C_{16:1}$	39.9	24.3	37.0	23.0	49.8	46.5
$C_{17:0\Delta}$	1.2	21.5	1.0	19.4	3.1	2.3
$C_{18:0}$	0.4	0.6	0.5	1.2	—	0.2
$C_{18:1}$	27.2	24.0	26.6	22.6	26.8	27.4
$C_{19:0\Delta}$	—	1.3	—	1.1	—	—
$C_{20:0}$	—	0.7	—	0.3	—	—
Unsaturated acids	68.3	71.1	64.6	66.1	79.7	76.2
Saturated acids	31.7	28.8	35.4	33.9	20.2	23.7
$C_{16:1}+C_{17:0\Delta}$	41.1	45.8	38.0	42.4	52.9	48.8
$C_{18:1}+C_{19:0\Delta}$	27.2	25.3	26.6	23.7	26.8	27.4

unsaturated acids. The proportion of unsaturated acids (including cyclopropane acids) in the extractable lipid did not vary significantly during the growth cycle, but was markedly affected by decreasing the growth temperature (Table 2). The increase in unsaturation as the growth temperature decreased was the result of an increase in $C_{16:1}+C_{17:0\Delta}$ fatty acids, since $C_{18:1}+C_{19:0\Delta}$ fatty acids did not change significantly with temperature (Table 2). Table 3 shows the fatty acid compositions of phosphatidylethanolamine isolated from organisms grown at 22°C and harvested after 20h, during the post-exponential phase of growth (exponential growth ended at 18h) and after 48h, during the stationary phase. Table 3 also shows the fatty acid composition of phosphatidylethanolamine isolated from organisms grown at 5°C and harvested after 165h during the stationary phase. These phosphatidylethanolamines were used for the physical studies described below.

Physical studies of lipids. (a) Some effects of the degree of unsaturation on the properties of phosphatidylethanolamine. Fig. 3 shows surface pressure–area curves for phosphatidylethanolamine isolated from *P. fluorescens* grown for 48h at either 22°C or at 5°C, the unsaturated acids, including cyclopropane acid, being 66 and 76% of the total respectively (Table 3). The surface pressure–area curve for phosphatidylethanolamine with 76% unsaturated acids shows a small expansion (cf. Haest, de Gier & van Deenen, 1969). The more expanded curve is similar to that for synthetic dioleoyl phosphatidylethanolamine (Chapman, Owens & Walker, 1966). Significantly,

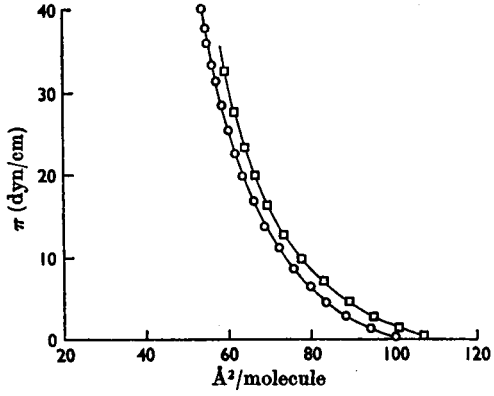

Fig. 3. Surface pressure–molecular area curves for phosphatidylethanolamine extracted from *P. fluorescens* on 0.1 M-NaCl at 22°C and 5°C. ○, *P. fluorescens* grown at 22°C, 66% unsaturated fatty acids; □, *P. fluorescens* grown at 5°C, 76% unsaturated fatty acids.

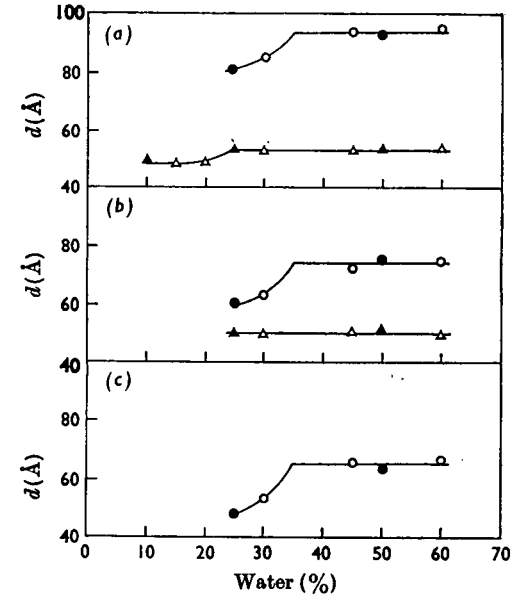

Fig. 4. Phase behaviour in water of phosphatidylethanolamine extracted from *P. fluorescens*. The limiting dimensions (d_{100}-lamellar and d_{10}-hexagonal) are compared at fixed temperature differences (ΔT) above the growth temperature: (*a*) at growth temperature $\Delta T = 0$°C, (*b*) at ΔT 23°C, (*c*) at ΔT 43°C (△, $d^{22°C}$ lamellar; ○, $d^{22°C}$ hexagonal; ▲, $d^{5°C}$ lamellar; ●, $d^{5°C}$ hexagonal. The superscripts refer to growth temperature.).

these isotherms remained unchanged, within experimental error, when the temperature was lowered to 5°C, which is again consistent with the general observation that when the chains are well above their melting point they are insensitive to temperature changes. However, if the experimental temperature is close to the liquid–crystalline transition temperature for such phospholipids then phase changes can be induced by relatively small variations in temperature.

The phase behaviour of phosphatidylethanolamine from organisms grown at 22°C and 5°C has been studied by X-ray diffraction methods. For the phosphatidylethanolamine extracted from *P. fluorescens* grown at 22°C for 24h, samples containing 0, 15, 20, 30, 45 and 60% of water were examined. At 22°C samples containing more than 20% water produced a mixed-phase system, lamellar+hexagonal, the limiting dimensions being d_{100} (lamellar) = 52.3 Å and d_{10} (hexagonal) = 94 Å (see Fig. 4*a*). It is not possible to determine the relative amounts of the two phases present. At lower water contents a lamellar phase only was present and in the absence of water the phosphatidylethanolamine gave a diffuse X-ray-diffraction pattern which probably arose from a lamellar phase. On increasing the temperature of the fully hydrated mixed-phase systems, the lamellar phase was gradually converted into the hexagonal phase until at 65°C the hexagonal phase alone was present. There did not appear to be a sharp transition from the lamellar to the hexagonal phase (cf. Reiss-Husson, 1967). For these samples there is calorimetric evidence that a gel–liquid crystal phase transition occurs at 10–15°C. At low water content (15 and 20%) no phase transition to the hexagonal phase occurred on raising the temperature.

For the phosphatidylethanolamine extracted from organisms grown at 5°C, samples containing 10, 25 and 50% water were examined. Once again at the growth temperature, 5°C, the samples with the higher water-content (25% and 50%) exhibited a mixed-phase system, lamellar+hexagonal; in this case the dimensions were d_{100} (lamellar) = 52.5 Å and d_{10} (hexagonal) = 92 Å (see Fig. 4*a*). For these two samples as the temperature was raised the lamellar phase was gradually converted into the hexagonal phase; for the 50% water sample the hexagonal phase only was present at 43°C. At low water content the lamellar phase was present and no obvious phase transition occurred as a function of temperature.

It is apparent that the bulk phase behaviour of the two phosphatidylethanolamine–water systems are related and, although changes occur in the lattice parameters as a function of temperature, there appears to be a direct relationship between the structural parameters and the growth temperature, particularly when excess of water is present. Fig. 4(*a*) shows that the diffraction patterns of the two systems at their growth temperatures, as a function

of water content, are superimposable. This agreement, both in terms of the phases present and their dimensions, suggests identical phase behaviour at the growth temperature. A similar effect is observed if comparisons are made at fixed temperature differences (ΔT) above growth temperature. Figs. 4(*b*) and 4(*c*) compare the results obtained with ΔT = 23° and 43°C. Two phases are present but at ΔT = 43°C the hexagonal phase only is observed, in both cases the phase behaviour being directly comparable.

Thus in some respects it appears possible to combine the structural information obtained from the two lipids providing that allowance is made for the difference in growth temperatures. This has been used to differentiate between the two hexagonal phases H_I (lipid cylinders in a water matrix) or H_{II} (water cylinders in a lipid matrix). At ΔT = 43°C the dependence of S, the surface area/lipid molecule at the lipid–water interface, on the lipid concentration suggests that the H_{II} phase is present. This is typical of diacyl lipids, although the H_{II} phase is usually found at the low water content part of the phase diagram (see Luzzati, 1968).

(b) Some physical properties of cyclopropane and unsaturated fatty acids. Figs. 5 and 6 show surface pressure–molecular area curves for a series of octadecanoic and octadecenoic acids at 22°C and 5°C respectively. The acids were all spread on 0.01M-hydrochloric acid so that ionization of the carboxyl groups was suppressed. To compare the effects of chain branching, the results of Ries & Cook (1954) for 16-methylheptadecanoic acid on water (pH 6) have been included in Fig. 5. Differences in the packing of the molecules arise from variations in the van der Waals forces between the alkyl chains. The isotherms of Figs. 5 and 6 show reasonable agreement with earlier reports where they are available, as reviewed by Gaines (1966). Although the measurements were made under a variety of conditions it is apparent from these early studies that, qualitatively, increasing amounts of unsaturation give rise to more-expanded isotherms. Also chain branching or unsaturation near the middle of an alkyl chain appears to decrease the intermolecular dispersion forces more drastically than when such substitution is near the ends of the chain.

The isotherms of Fig. 5 indicate that the packings of fatty acids containing a *cis*-9,10-methylene group, *trans*-9- or *cis*-6-unsaturated bonds are similar at 22°C. A *cis*-9- double bond gives the greatest expansion whereas substitution of a methyl group on the C-16 position leads to relatively little disruption of the chain packing. Under the conditions specified in Fig. 5 only the fully saturated chains can crystallize and all the other chains are melted. Cooling the monolayers to 5°C has significant effects upon the petroselinic and elaidic acid

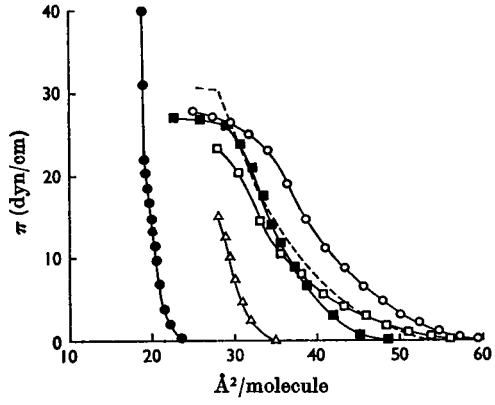

Fig. 5. Surface pressure–molecular area curves for some octadecanoic and octadecenoic acids and cyclopropane acids on 0.01M-HCl (pH 2) at 22°C. ●, Stearic acid; □, elaidic acid; ----, petroselinic acid; ■, dihydrosterculic acid; ○, oleic acid. △, 16-Methylheptadecanoic acid on water (pH 6) (Ries & Cook, 1954).

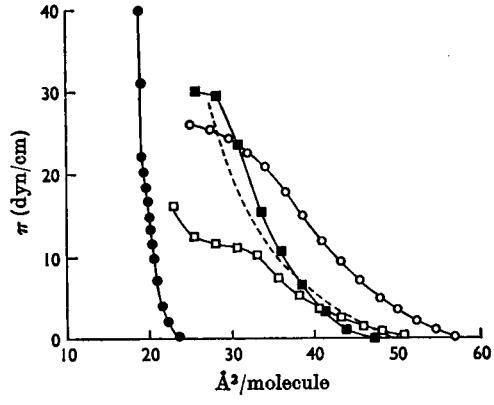

Fig. 6. Surface pressure–molecular area curves for some octadecanoic, octadecenoic and cyclopropane acids on 0.01M-HCl (pH 2) at 5°C. Symbols are as in Fig. 5.

films only. The petroselinic acid becomes more close-packed but remains liquid whereas the elaidic acid chains undergo a two-dimensional crystallization.

Table 4 lists the latent heats of fusion of a series of octadecanoic, octadecenoic and cyclopropane acids. Where comparisons can be made the results are close to those reported by Bailey (1950) and Lutton (1967). It is clear that the sequence parallels that of the observed interfacial areas at high surface pressures. Interpretation of these thermodynamic parameters is complicated by the well-known polymorphism of fatty acids which makes it difficult to ensure that they have all

Table 4. *Thermodynamic parameters for fusion of some octadecanoic, octadecenoic and cyclopropane acids*

Fatty acid	Melting temperature (T°C)	Enthalpy change (ΔH) (kcal/mol)	Entropy change (ΔS) (cal·deg^{-1}·mol^{-1})
Octadecanoic (stearic)*	69.6	16.36	47.5
trans-9-Octadecenoic (elaidic)	45	13.7	43
cis-6-Octadecenoic (petroselinic)	30	11.7	39
cis-9,10-Methyleneoctadecanoic (dihydrosterculic)	38	9.9	32
cis-11-Octadecenoic (*cis*-vaccenic)	12	9.3	33
cis-9-Octadecenoic (oleic)	−9	1.7	6
	13	8.1	28

* Results from Singleton, Ward & Dollear (1950).

adopted the same crystal form. Thus our sample of oleic acid underwent two thermal transitions, the change at −9°C probably being due to some pre-melting phenomenon. However, the enthalpies of fusion do provide a measure of the inter-chain van der Waals forces (Phillips, Williams & Chapman, 1969). The introduction of a *cis*-double bond into stearic acid drastically decreases the melting point and almost halves the chain interaction energies; the effect being greatest when the unsaturation is near the middle of the chain.

Taken together the monolayer and fusion results indicate that for 9,10-substituted octadecanoic acids the intermolecular apolar interactions decrease in the order stearic > elaidic > dihydrosterculic > oleic. Dihydrosterculic acid behaves more like elaidic than oleic acid in terms of chain packing.

The phosphatidylethanolamine isolated from *P. fluorescens* after 20 h growth at 22°C contains about 1% cyclopropane acid whereas after 48 h the amount has increased to almost 20%. There is only a small change in the total amount of unsaturation (see Table 3) if the cyclopropane acids are counted as unsaturated. The surface pressure–area isotherms of these phosphatidylethanolamines are the same, indicating that in these phospholipids the effects on the chain packing of converting *cis*-double bonds to cyclopropane rings are insignificant (cf. Demel, 1966; Haest *et al.* 1969). Thus, providing the surface properties of these lipids do reflect their packing properties in membranes, accumulation of cyclopropane acids by an organism will not greatly alter the physical properties of the membrane. The above results also indicate that the practice of calculating unsaturated/saturated fatty acid ratios by taking the cyclopropane acids as unsaturated is physically meaningful and this practice will be adopted for the purposes of the discussion.

DISCUSSION

The increase in the proportion of unsaturated lipids, or in the degree of unsaturation of fatty acids as a result of a decrease in environmental temperature, has been described for a variety of biological systems and is not peculiar to *P. fluorescens*. Where unsaturated acids are formed by oxidative desaturation of long-chain saturated acids, the effect of variations in the availability of oxygen on the degree of unsaturation has been examined. Harris & James (1969) showed that in bulb tissue the effect of decreasing the temperature was to increase the availability of oxygen, the concentration being rate-limiting for the synthesis of unsaturated acids. Jollow, Kellerman & Linnane (1968) described marked differences in the fatty acid compositions of *Saccharomyces cerevisae* grown in aerobic and anaerobic conditions, anaerobic growth resulting in a decrease in synthesis of unsaturated acids. Brown & Rose (1969) showed that in *Candida utilis* the effects of decreasing the temperature and increasing the dissolved-oxygen partial pressure were separate and similar.

An increase in unsaturation accompanying a decrease in temperature has also been described in *Serratia marcescens* (Kates & Hagen, 1964) and *Escherichia coli* (Marr & Ingraham, 1962; Shaw & Ingraham, 1965; Knivett & Cullen, 1965; Haest *et al.* 1969). *P. fluorescens* is an aerobe and *S. marcescens* and *E. coli* are facultative anaerobes, but it is likely that all three organisms synthesize unsaturated acids by an anaerobic pathway (Scheuerbrandt & Bloch, 1962; Erwin & Bloch, 1964). The pathway proposed for this anaerobic synthesis has been the subject of detailed study, but the mechanism of the temperature-sensitive control of the unsaturated/saturated fatty acid ratio is not known (Kass, Brock & Bloch, 1967; Birge, Silbert & Vagelos, 1967; Helmkamp & Bloch, 1969). The effect of decreasing the growth temperature on the synthesis of unsaturated acids in such a system is unlikely to be directly related to increased oxygen availability. In *E. coli* the proportion of unsaturated acids (including cyclopropane acids) did not vary with oxygen supply (Knivett & Cullen, 1965).

In various bacteria cyclopropane acids accumulate after the end of exponential growth (Law, Zalkin & Kaneshiro, 1963; Marr & Ingraham, 1962; Knivett & Cullen, 1967). Under our conditions the amount of cyclopropane acid in *P. fluorescens* growing exponentially was less than 1% of the fatty acids at all temperatures examined and the conversion of unsaturated acid into cyclopropane acid was apparently unaffected by the events of the phase of decline. Cyclopropane acid accumulation was, however, affected by temperature (Figs. 1 and 2). A decrease in accumulation of cyclopropane acids at lower growth temperatures was described in *S. marcescens* (Kates & Hagen, 1964) and in *E. coli* (Knivett & Cullen, 1965). Cyclopropane acid accumulation was also affected by oxygen supply in *E. coli* (Knivett & Cullen, 1965; Crowfoot & Hunt, 1970) and in *P. fluorescens* (Crowfoot & Hunt, 1970) where cyclopropane acid concentrations were inversely proportional to oxygen partial pressure, indicating that cyclopropane acid accumulation in batch cultures, where aeration is low, may be related to a decrease in oxygen partial pressure. Under the conditions described here no significant increase in cell yield was observed with a decrease in growth temperature, indicating that availability of oxygen was not limiting for growth at the higher temperatures.

Whatever the biochemical reasons for the increase in unsaturation of lipids with decrease in environmental temperature observed in this system (and many others), it is important to consider the physicochemical consequences of such changes. Since in *P. fluorescens* variations in temperature do not significantly affect the distribution of phospholipid classes and only alter the hydrocarbon chain composition, it is the physical properties of hydrocarbon chains that are particularly relevant.

In a model membrane system composed of phospholipid liposomes the bilayer permeability is strongly dependent on the alkyl chain structures (de Gier, Mandersloot & van Deenen, 1968). The permeability can be enhanced when the chain packing density, as inferred from monolayer studies, is decreased either by introducing double bonds or by a decrease in chain length. However, in membranes, the lipid packing will be modified by interaction with the remaining components, but there is evidence for a relationship between the fatty acid composition and permeability of erythrocyte membranes (Kogl, de Gier, Mulder & van Deenen, 1960; Walker & Kummerow, 1964). Such permeability effects have been invoked as a cause of the increase in the amount of unsaturated fatty acids with decrease in growth temperature of micro-organisms. It has also been suggested that to maintain its permeability properties, a micro-organism controls the hydrocarbon chain packing of its membrane. In support of this, a detailed study of an unsaturated fatty acid auxotroph of *E. coli* (Esfahani, Barnes & Wakil, 1969) suggested the operation of a regulatory mechanism that controls the composition of saturated versus unsaturated acids to maintain the physical properties of phospholipids within narrow limits.

We have shown that an increase in the degree of unsaturation of bacterial phosphatidylethanolamine results in an expansion of the monolayer (see Fig. 3). Haest *et al.* (1969) showed that an increase in permeability of liposomes prepared from the lipids of *E. coli* resulted from increased unsaturation and tentatively concluded that the bacteria compensated for a decreased growth temperature by increasing the degree of hydrocarbon chain unsaturation. However, since the molecular packings at the air–water interface of phosphatidylethanolamine with either 66% or 76% of its fatty acids unsaturated were essentially insensitive to a temperature decrease of 17°C (Fig. 3) it appears that, within a given membrane structure, the permeability would remain unchanged over such a temperature range without any changes in unsaturation. In order to determine whether changes in the permeability of artificial membranes prepared from bacterial lipids parallel the organism's functional requirements, it will be necessary to seek correlations between the temperature-dependence of the leak rates from such liposomes and the growth temperature of the organism from which the lipids are obtained.

Another possibility is that structural re-organizations within membranes are employed as a means of controlling permeability and it is here that the bulk-phase properties of the isolated lipids are relevant. It is striking that the organism grown at 22°C produced lipids that exhibited a certain phase behaviour at 22°C and when the organism was grown at 5°C it produced lipids that gave this same behaviour at 5°C with the same characteristic dimensions (Fig. 4*a*). However, on raising the temperature of the latter sample to 22°C, although the same phases were present, they had different characteristic dimensions. These dimensions d, the lamellar repeat or distance between rows of cylinders, and hence S, area/lipid molecule, are temperature dependent and changes in surface area would seem to conflict with the monolayer behaviour which indicates only a small change in molecular area on changing the temperature by 17°C. However it is reasonable to compare the monolayer studies only with the lamellar phase (Phillips & Chapman, 1968) which is much less temperature dependent than the hexagonal phase (Fig. 4).

Although it is unlikely that this mixed-phase system is present in the membrane it appears that

there is some control in the organism that dictates that the fatty acids produced at 22°C or 5°C, for example, when incorporated into lipids, do have characteristic and identical physical properties at the temperatures at which they will operate. As might be expected the overall effect of increasing the amount of unsaturation is to shift the phase diagram down the temperature axis. It is noteworthy that the liquid–crystalline transition temperature range of both the isolated lipids and the whole membranes of *Mycoplasma laidlawii* changed so that the growth temperature was encompassed when the organism was grown at 25° and 37°C (Melchior, Morowitz, Sturtevant & Tsong, 1970).

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