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THE BIOSYNTHETIC INCORPORATION OF DIACETYLENIC FATTY ACIDS INTO THE BIOMEMBRANES OF *ACHOLEPLASMA LAIDLAWII* A CELLS AND POLYMERISATION OF THE BIOMEMBRANES BY IRRADIATION WITH ULTRAVIOLET LIGHT

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***Acholeplasma laidlawii* A has been grown in media containing synthetic, long chain C_{20}- and C_{23}-fatty acids possessing a diacetylene group in their acyl chains. Growth on the C_{23} diacetylenic acid was poor but was good on the C_{20} acid. Biosynthetic incorporation of the fatty acids occurs; as much as 90% of the membrane lipid fatty acyl chains consisting of the C_{20}-diacetylenic fatty acid, the remainder being shorter chain, saturated fatty acids. The thermal phase transition of this biomembrane has been studied and a differential scanning calorimetry heating curve shows the presence of an endotherm corresponding to a membrane lipid phase transition occurring at about 26°C. The lipid class composition of membranes containing the C_{20}-diacetylene lipids was examined and found to be similar to membranes from cells grown on oleic acid-containing medium. (The ratio of monoglucosyl- to diglucosyldiacylglycerols was the same but the ratio of glycolipid to phosphatidylglycerol was higher in the cells grown with diacetylene fatty acids). Upon irradiation with ultraviolet light the cells and isolated biomembranes become coloured, either red or yellow depending upon their thermal history. The colour change indicates that extensive cross-linking of the lipids of the biomembranes of *A. laidlawii* has occurred and that a conjugated polymeric structure has been formed. Analysis of the extracted lipids from the biomembranes by GLC indicates that extensive cross-linking of the lipid chains within the biomembrane of a natural cell system has been achieved. The monoglucosyldiacylglycerols cross-link more readily that do the phosphatidylglycerol lipids. The effect of such lipid cross-linking or polymerisation on the activity at 35°C of an intrinsic membrane-bound enzyme, NADH oxidase, and ribonuclease, an extrinsic membrane-bound enzyme, was studied. The NADH oxidase activity decreased rapidly upon cross-linking of the lipid environment whereas ribonuclease activity was unaffected. The potential for future studies of polymerised model and natural biomembranes is discussed.**

Introduction

Cell membranes are composed of a lipid bilayer with which are associated proteins of an intrinsic and extrinsic nature. The activity of the intrinsic proteins can be dictated to a greater or lesser degree by the physical state of the lipid bilayer matrix [1]. A number of methods have been used to study the dependence of protein function on lipid composition. These include the reconstitution of enzymes in liposomes having a defined composition [2] and the manipulation of cell membranes by hydrogenation of unsaturated lipids [3], fusion of liposomes with membranes [4] or by the use of cell strains which due to enzyme defects are dependent upon their growth medium for the supply of the fatty acids from which their lipids are synthesised.

The simple bacterium *A. laidlawii* has proved very useful in the production of membranes with defined composition. Due to the lack of the enzyme β-hydroxydecanoate dehydrase, the organism is an unsaturated fatty acid auxotroph. This has allowed extensive manipulation of both the unsaturated and saturated fatty acids composition of the cell membrane of this organism. Furthermore, the cells lack a wall, being bounded solely by the plasma membrane which also constitutes the only membrane of the cell. A great deal of work involving the manipulation of *A. laidlawii* membranes has been performed [5–8] and the effect of fatty acid composition on properties such as permeability [9], cell morphology [10] and enzyme activity [11,12] has been studied.

Recently, we have reported from our laboratory the synthesis of fatty acids and phospholipids which contain diacetylenic groupings. These molecules when irradiated in mono- and multilayers with ultraviolet light cross-link via the diacetylene groupings forming polymers which, due to the conjugated nature of their backbone, are highly coloured [13]. Liposomes of these phospholipids have also been polymerised producing a red coloured polymer [14].

In the present paper we report the biosynthetic incorporation of diacetylenic fatty acids into the biomembranes of *A. laidlawii* cells and their subsequent polymerisation in situ. In order to determine the effect of polymerisation on the properties of the membrane, we have also studied the activity of an intrinsic membrane-bound enzyme, NADH oxidase (EC 1.6.99.3) and an extrinsic membrane-bound enzyme, ribonuclease [15]. The activity of NADH oxidase has previously been studied in *A. laidlawii* membranes [16] and its purification from *A. laidlawii* was recently described [17]. Some preliminary observations on the effect of polymerisation on the activity of these enzymes are included in this paper.

Materials and Methods

Materials

A. laidlawii strain A(NCTC 10116), isolated from sewerage, was obtained from the National Collection of Type Cultures, London.

n-Eicos-10,12-diynoic acid (C_{20} diacetylenic acid), *n*-tricos-10,-12-diynoic acid (C_{23} diacetylenic acid) and their respective phospholipids were synthesised as detailed in earlier studies [14].

Methods

Bacterial culture. A. laidlawii were grown statically at 37°C in 2-litre conical flasks (containing 1 litre of medium) tilted at an angle so as to increase the surface area and aid aeration. One litre of medium contained: 5 g Tris base; 20 g tryptose (defatted with chloroform/methanol and diethyl ether); 5 g NaCl; 4 g bovine serum albumin (defatted with acetone); 5 g glucose; 60 mg penicillin. The pH was 8.2. The fatty acid (120 μmol) was added to this as an ethanolic solution, the final concentration of ethanol being 0.1% (v/v). A 1% innoculum was used. Late exponential or stationary phase bacteria were harvested by centrifuging at $9000 \times g$ and 2°C for 10 min. They were then washed twice with a large excess of 0.25 M NaCl to remove any residual free fatty acid.

Membrane isolation. Membranes were prepared and isolated from late exponential cultures. Cells were ruptured by osmotic shock and the membranes isolated by differential centrifugation as detailed by Rottem et al. [18]. The percentage lysis of the cells was 80% or better. The membranes were finally resuspended in sufficient buffer to give a protein concentration of about 5 mg/ml and stored frozen.

Determination of lipid composition. The extraction of lipids, fractionation into classes and the preparation of the methyl esters of the fatty acids were all performed in a similar manner to that detailed by Saito and McElhaney [6]. The methyl esters of the fatty acids were separated on a SE-30 column using a Pye Unicam gas-liquid chromatograph.

Membrane lipid head group composition. Lipids extracted from the membranes of cells grown in the presence of various fatty acids were separated into their head group classes by thin-layer chromatography and quantified as detailed by Silvius et al. [12]. *n*-Heptadecanoic acid (50 μg) was used as an internal standard.

Irradiation of samples. Whole cells (resuspended in 0.25 M NaCl) membranes and extracted lipids (in the form of liposomes prepared by vortexing with water above their transition temperature),

were irradiated at 2°C after very thorough purging with nitrogen, using a Mineralight R-52 lamp. This lamp had a peak radiation intensity at 254 nm and an energy output of 1200 $\mu W/cm^2$ at 15.2 cm from its face.

The intensity of the ultraviolet light was varied by changing the distance between the light source and the cuvette and the incident energy calculated using the inverse square law.

The degree of cross-linking of the lipids was determined by gas-liquid chromatography. The area of the peaks of the methyl esters of the diacetylenic fatty acids present in the membrane, before and after irradiation, was compared with that of an oleic acid standard, the oleic acid being added to the cell or membrane suspensions after irradiation but before lipid extraction.

Measurement of NADH oxidase activity. NADH oxidase activity was measured by following the decrease in absorbance at 334 nm using oxygen as electron acceptor as described by Reinards et al. [17]. The reaction mixture (3 ml) contained 0.1 M Tris HCl (pH 7.4) and 0.169 M NADH. The activity was assayed at 35°C and was initiated by the addition of membrane.

Measurement of ribonuclease activity. Ribonuclease activity at 35°C was determined by measuring the absorbance at 260 nm of soluble nucleotides released from RNA as detailed by Ne'eman and Razin [15]. The dithiothreitol concentration used was 0.5 mM.

Differential scanning calorimetry (DSC). DSC curves of whole membranes was performed on samples containing 2 mg of protein. Thermal analysis of the extracted and pure lipids were performed on 10 mg samples suspended in 50 μl of water. Sensitivity was 0.5 mcal/s and the scan rate was 5 K/min. In both cases a Perkin Elmer DSC 2 calorimeter was used.

Spectra. Difference spectra of the membranes and extracted lipids before and after polymerisation were obtained using the Shimazu MPS 504 spectrophotometer of London University's School of Ophthalmology.

Results

The growth of *A. laidlawii* on either C_{20} diacetylenic, oleic or palmitic/myristic acids was similar. Stationary phase was reached after 48 h and the yield of cells was about 250 to 300 mg/l wet weight. Growth on C_{23} diacetylenic fatty acids was poor, the yield of cells being less than 50 mg/l.

GLC analysis of methyl esters of the extracted lipids showed that about 90% of the fatty acyl chains of cells grown on C_{20} diacetylenic or oleic acid were derived from the medium, the remainder being shorter chain saturated fatty acids (Table I). Lipids extracted from cells grown on C_{23} diacetylenic acid contain only 40 to 45% of exogenous acid. Analysis of the phospholipid and glycolipid fractions of the extracted lipids from the cells grown on C_{20} diacetylenic acid showed that their fatty acid composition was identical with that of the total cell lipid extract indicating that there was no preferential incorporation of the diacetylenic acid into any particular lipid class.

The lipid headgroup composition of membranes from cells grown on C_{20} diacetylenic fatty acid was similar to that of cells grown on oleic acid (Table II). The ratio of monoglucosyl- to diglucosyldiacylglycerol was the same but the ratio of glycolipids to phosphatidylglycerol was higher in the C_{20} diacetylene grown cells than in oleate grown (1.6 compared with 1.13). This was still much lower than in cells grown on saturated palmitic/myristic acids (3.3). Trace amount of aminoacyl phosphatidyl glycerol were present in cells grown with the C_{20} diacetylene fatty acid.

Scanning calorimetric heating curves of membranes from cells grown on C_{20} diacetylenic fatty acid show a broad transition beginning at 26°C (Fig. 1). DSC curves of the total extracted lipids showed a transition at 27.5°C and the transition of synthetic C_{20} diacetylenic lecithin was 22°C (see Fig. 1). As with C_{20} diacetylenic phosphatidylcholine liposomes, no transition is observed after 5 min irradiation (Leaver, Alonso, Durrani and Chapman, in preparation).

Whole, freshly harvested and washed cells grown on either C_{20} or C_{23} diacetylenic acids did not when irradiated undergo any dramatic colour changes. However, hydrated lipids extracted from cells grown on C_{20} gave a pale yellow colour as a result of irradiation. The ultraviolet spectra of hydrated lipids before and after irradiation were similar to that previously observed with liposomes

TABLE I

FATTY ACID COMPOSITION OF TOTAL LIPIDS OF *A. LAIDLAWII* GROWN IN THE PRESENCE OF VARIOUS FATTY ACIDS

See text for experimental details. DA, diacetylenic acid.

Fatty acid in medium	Composition (mol %)						
	C_{20}DA	C_{23}DA	Oleic	Stearic	Palmitic	Myristic	Others
C_{20}DA	90	–	–	–	–	–	10
C_{23}DA	–	39	–	8	18	19	16
Oleic	–	–	90	–	6	3	1
Palmitic/Myristic (1 : 1, mol/mol)	–	–	–	–	39	45	16

TABLE II

LIPID HEADGROUP COMPOSITION OF MEMBRANES OF *A. LAIDLAWII* CELLS GROWN IN THE PRESENCE OF VARIOUS FATTY ACIDS

Experimental details are described in the text. All membrane samples has been stored frozen for not more than 10 days. Samples were irradiated at 0°C for 5 min prior to extraction. Palmitic/myristic acid concentrations were 1 : 1 (mol/mol).

Fatty acid in medium	Headgroup composition (mol %)				
	Monoglucosyl-diacylglycerol	Diglucosyl-diacylglycerol	Phosphatidyl-glycerol	Glycerophosphoryl-diglucosyldiacyl-glycerol	3'-*O*-Aminoacyl-phosphatidyl-glycerol
Oleate	22.2	21.7	38.8	7.7	9.6
Palmitic/ Myristic	26.6	39.0	19.9	10.3	4.2
C_{20}DA	29.4	29.4	35.4	4.8	–
C_{20}DA Irradiated	18.0	33.5	45.8	2.6	–

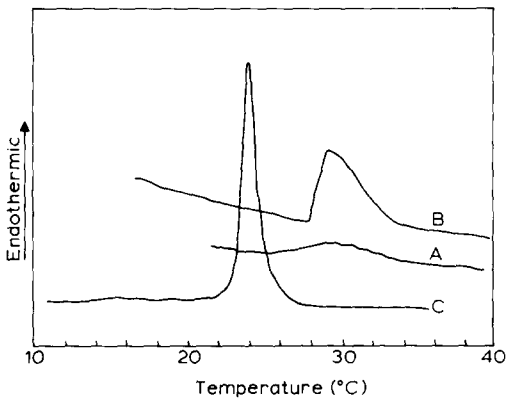

Fig. 1. Heating calorimetric curves of whole membranes and a total lipid extract of *A. laidlawii* cells grown on C_{20} diacetylenic fatty acid and liposomes of C_{20} diacetylenic phosphatidylcholine. (A) Membrane sample containing 2 mg protein. (B) Extracted membrane lipids containing 10 mg lipid in 50 μl water. (C) C_{20} phosphatidylcholine containing 10 mg lipid in 50 μl water. Sensitivity = 0.5 mcal/s; scan rate = 5 K/min.

of synthetic C_{23} phosphatidylcholine [14]. Extracted lipids from cells grown on C_{23} did not produce any coloured polymer when irradiated.

When cells grown on C_{20} diacetylenic acid were stored at −30°C for 24 to 48 h with or without the addition of glycerol to prevent freezing and the cells were irradiated at a temperature of 2°C, the colour of the cell suspensions changed dramatically from white to red. Similarly, hydrated lipid extracts, irradiated at 2°C before or after storage at −30°C, gave, respectively, pale yellow or red coloured material. Storage at low temperature prior to irradiation favours the production of the red-coloured material. Irradiation above about 4°C resulted in the formation of yellow coloured polymer. The difference spectrum of whole *A. laidlawii* cells before and after irradiation is shown in Fig. 2

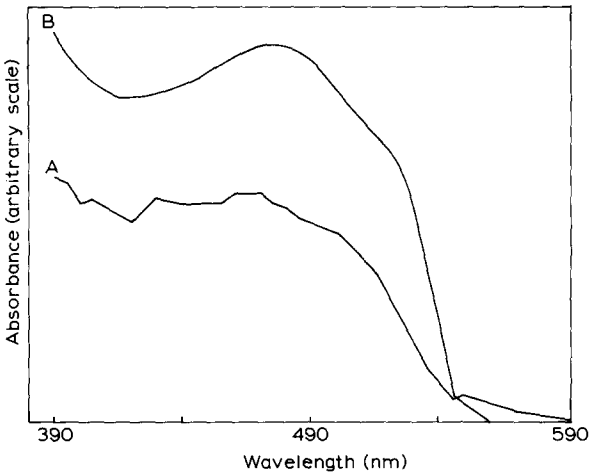

Fig. 2. Visible difference spectra of (A) polymerised whole *A. laidlawii* cells grown on C_{20} diacetylenic fatty acid and (B) polymerised C_{20} diacetylenic liposomes. Cells and liposomes were irradiated at 2°C for 5 min and recorded at 20°C. After harvesting and washing cells were stored for 48 h at −30°C prior to irradiation. Intensity of UV irradiation was 0.27 W/cm^2.

and can be compared with the spectrum of polymerised liposomes of C_{20} diacetylenic phosphatidylcholine also shown in Fig. 2.

The effect of the irradiation period on the degree of cross-linking in whole cells is shown in Fig. 3. For curve A, the cells were irradiated for short periods up to the time indicated. The samples irradiated for a total period of 15, 30 and 60 s were each irradiated in 15-s bursts and the remaining samples in 30-s bursts. For curve B, the samples were irradiated continuously for the indicated time. When cells which were irradiated for a total time of 10 min were examined, those irradiated for 30 s periods had a maximum of 38% of the C_{20} diacetylenic acid cross-linked whereas those irradiated continuously had 73% cross-linked. The latter value corresponds to approximately 66% of the total membrane lipids. As much as 70 to 97% of the diacetylene chains could be cross-linked depending on the batch of cells and storage conditions. Those cells which were irradiated in short bursts were found to cross-link to the same degree (about 40%) irrespective of whether they had been stored at low temperatures or not, and even freshly harvested cells which did not produce any obvious colour change were found to contain up to 40% cross-linked diacetylenic fatty acyl chains. Similar results were obtained with isolated membranes.

Table II shows that cross-linking of the diacetylene-containing acyl chains of glycolipids, particularly monoglucosyldiacylglycerol occurred more readily than did that of phosphatidylglycerol chains as judged by the change in the proportions of each lipid class following irradiation.

The effect of the intensity of the ultraviolet irradiation on the amount of polymer formed in whole membranes is shown in Fig. 4. The degree of cross-linking was linear with respect to the logarithm of the intensity over the range tested. With cell suspensions irradiated for long periods

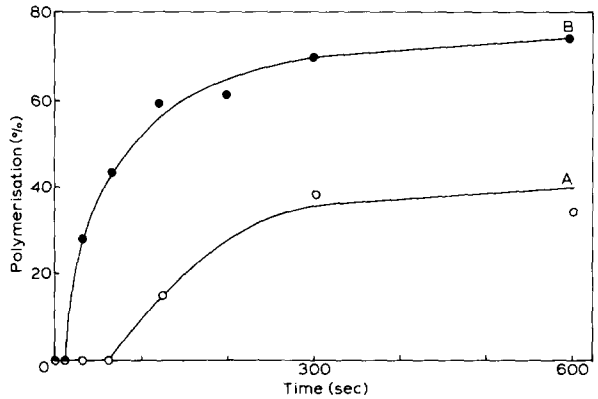

Fig. 3. Effect of irradiation time on the polymerisation of whole *A. laidlawii* cells grown on C_{20} diacetylenic fatty acid. (A) Irradiated in short bursts (see text for details). (B) Irradiated continuously. Cells were stored and irradiated as detailed in Fig. 2.

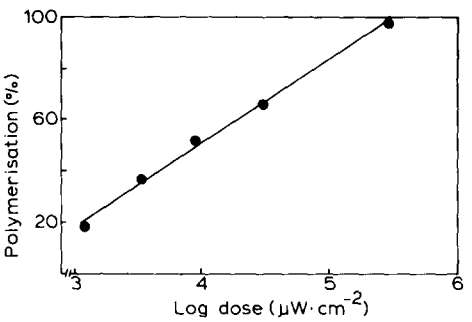

Fig. 4. Effect of the intensity of ultraviolet light on the degree of polymerisation of whole *A. laidlawii* grown on C_{20} diacetylenic fatty acid as a function of the log of the intensity. Irradiation was for 5 min at 2°C. Cells were stored as detailed in Fig. 2.

and at high intensities a 'bleaching' effect occurred where the intensity of the colour decreased as a result of prolonged exposure to the ultraviolet light.

Red coloured polymer was found to be mainly insoluble in the chloroform/methanol mixture used to extract the lipids. Traces of colour were occasionally seen in highly concentrated chloroform extracts. In the presence of chloroform, the colour changed to yellow.

The molar extinction coefficient of the red polymer in membranes was estimated to be approx. $1.35 \cdot 10^2\ l \cdot mol^{-1} \cdot cm^{-1}$ at 460 nm on the basis of the absorbance of the irradiated preparation and the amount of monomer cross-linked as measured by GLC.

The effect of polymerisation on the activity of NADH oxidase, an intrinsic protein, and ribonuclease, an extrinsic protein, was studied. To determine the effect of irradiation with ultraviolet light on the activity of the enzyme in a non-polymerisable membrane, the activity of these enzymes in C_{20} diacetylenic fatty acid-containing membrane was compared with that of the enzymes in oleic acid membranes.

Frozen storage did not affect the activity of the enzymes, but membranes obtained from cells

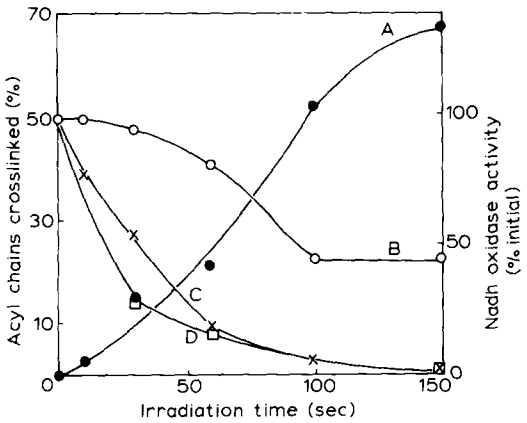

Fig. 5. The effect of the length of the irradiation period on the NADH oxidase activity in C_{20} diacetylene- and oleic acid-containing membranes and on the degree of cross-linking of the diacetylene-containing acyl chains. (A) Cross-linking. (B) NADH oxidase activity in oleic acid-containing membrane (Average from three preparations). (C/D) NADH oxidase activity in C_{20} diacetylenic fatty acid-containing membrane from two preparations. Membranes were irradiated at 2°C after storage at −30°C for 48 h.

grown on oleic acid when irradiated rapidly lost NADH oxidase activity. The addition of dithiothreitol, a sulphydryl protecting agent, not only greatly increased the initial activity of this enzyme but also increased its stability to irradiation with ultraviolet light. Dithiothreitol did not have any significant effect on the degree of polymerisation of diacetylenic phospholipid liposomes and it was therefore included in all samples used for NADH oxidase studies to a final concentration of 20 mM.

The effect of the length of the irradiation period and hence the dose of ultraviolet light on NADH oxidase activity in membrane obtained from cells grown on oleic acid and on C_{20} diacetylenic acid, and also on the cross-linking of the C_{20} diacetylenic acyl chains is shown in Fig. 5. The rate at which the NADH oxidase activity decreased was much greater in the C_{20} diacetylenic fatty acid containing membrane than it was in the oleic acid-containing membrane. After 60 s irradiation the diacetylenic membrane lost over 80% of its NADH oxidase activity compared to 20% in the oleic acid membrane. After 150 s, the enzymatic activity in the diacetylene-containing membrane was lost completely whereas the oleate-membrane still retained over 40% of its original activity. The rat of loss of enzymatic activity in the polymerisable membrane correlated with a steady increase in the amount of the membrane lipid which was cross-linked. The increased loss of NADH oxidase activity is therefore due to polymerisation of the diacetylenic lipids. In the first 10 s of irradiation, the formation of a small amount of cross-linked lipid (less than 3%) resulted in a loss of more than 20% of the NADH oxidase activity.

Ribonuclease activity in membranes obtained from cells grown in the presence of both oleic acid and C_{20} diacetylenic acid was not affected by irradiation with ultraviolet light even when the concentration of dithiothreitol was as low as 0.5 mM. In complete contrast to the effects observed with the NADH oxidase, enzyme activity was not altered by either ultraviolet light irradiation or by polymerisation.

Discussion

Our previous studies with synthetic phospholipids containing diacetylene groups in their acyl

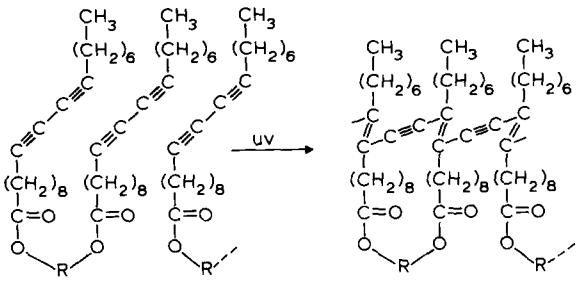

Fig. 6. Schematic representation of the formation of conjugated lipid polymer from C_{20} diacetylenic lipid monomer.

chains have shown that polymerisation forming highly coloured polymers can be induced by ultraviolet light irradiation (Fig. 6) when the lipids are arranged in monolayers, liposomes and also in multilayer forms, i.e. with various model biomembrane systems [13,14]. This raised the question: Can similar extensive cross-linking and polymer formation be induced in natural biomembranes? To answer this question, two requirements need to be satisfied. The first is the ability of a suitable cell system to biosynthetically incorporate appreciable amounts of a diacetylenic fatty acid into its biomembranes. The second arises from the fact that polymerisation of diacetylene lipids is favoured when a highly regular crystalline arrangement is present. Hence a cell which can contain extensive regions of organised lipid at a convenient temperature is needed. Both of these requirements are fulfilled by *Acholeplasma laidlawii* cells. McElhaney and co-workers [5,8] have demonstrated that extensive manipulation of the fatty acid composition of these cells can be made. Furthermore, various studies of phase transitions of such cell systems [19], demonstrate that extensive regions of crystalline lipid within the biomembranes can be readily induced by lowering the temperature of the cells. (The second requirement can be fulfilled by many cell biomembranes where cholesterol is absent.) A lowering of the temperature leads to lipid crystallisation and protein clustering (for review, see Ref. 1).

The fact that *A. laidlawii* cells meet both these requirements is therefore convenient for the present study. This is the first time that *Acholeplasma* cells have been grown on diacetylenic fatty acid and as we have shown, the amount of C_{20} diacetylenic acid incorporated into the membrane is very high compared with the amount of C_{23} diacetylenic fatty acid. The suitability of a particular fatty acid as a substrate for the growth of *A. laidlawii* has been shown to be dependent on the physical properties of the fatty acid. Those which give diacylglycerides with a very high or low fluidity at growth temperatures have been found to be poor substrates [8]. Membranes containing large amounts of C_{20} diacetylenic acid have a transition temperature of 26°C and therefore satisfy this requirement with cells grown at 37°C. Synthetic C_{23} diacetylene phosphatidylcholine has a transition temperature of 38°C and the membrane glycolipids containing solely C_{23} diacetylenic acyl chains are expected to have a much higher transition temperature [20].

Although the cell can compensate for changes in membrane viscosity by modifying the lipid head group composition, i.e. by increasing the monoglucososyldiglycerols at the expense of the diglucosyl component as 'viscosity' increases [20,21], it must also maintain a stable membrane bilayer. The monoglucososyldiglycerol has a lower phase transition than does the corresponding diglucosyldiglycerol [20] but tends to form a reversed hexagonal phase due to its wedge-shape, particularly if both chains are unsaturated. This is said to destabilise the membrane bilayer [22]. However, higher amounts of monoglucosyldiglycerols can be maintained if the cells are grown on a mixture of saturated and unsaturated fatty acids [21]. Presumably in this case one acyl chain of the lipid is saturated and the other unsaturated, thus reducing the wedge-shape of the molecule and hence its tendency to destabilise the bilayer. These considerations may be important with cells grown on the C_{23} fatty acid, but there are other effects to consider.

The good incorporation of C_{20} diacetylenic fatty acid together with the similarity of the lipid head distribution with both the C_{20} diacetylene- and the oleic acid-grown cells suggest that the C_{20} diacetylene-containing lipids have a sufficiently similar geometry to oleoyl-containing lipids and thus are arranged in the bilayer in a similar manner. The effect of lipid molecular geometry on lipid phase structure and membrane stability in *A. laidlawii* has been discussed by Wieslander et al. [22].

An additional factor which must be considered is the considerable length of the C_{23} fatty acid and the optimum requirements for matching the protein hydrophobic segment with the lipid chain lenght.

Extensive polymerisation of the membrane of cells grown on C_{20} diacetylenic fatty acid was induced by ultraviolet light irradiation. The results presented in this paper show that up to 97% of the diacetylenic fatty acyl chains (comprising 90% of the total lipids) of *A. laidlawii* membranes can be crosslinked. This is despite the heterogenity of the lipid headgroups and the large proportion of protein in the membrane (protein to lipid ratio of about 2 : 1 (w/w)). The glycolipids, particularly the monoglucosyldiacylglycerol component, appears to cross-link more readily than phosphatidylglycerol. This may indicate that glycolipids pack in more regular crystalline arrays than do the phospholipids.

The red-coloured polymer produced with the *Acholeplasma laidlawii* in biomembranes has properties very similar to that of polymerised phosphatidylcholine. The visible and ultraviolet spectra are very similar, as is the molar extinction coefficient and the effect of chloroform on the colour. Furthermore, multilayers of diacetylenic lecithins when irradiated for long periods have been observed to undergo 'bleaching' of the colour in the same way as the cell suspensions. The cause of this effect is as yet unknown.

The effect of storage conditions on the colour and hence the type of polymer produced in whole cells and liposomes is of interest. The red colour probably corresponds to a longer conjugated polymer that the yellow material. At low temperatures, the lipids, already in a crystalline phase, probably become more strictly orientated with respect to each other, hence leading to more efficient polymerisation. It is known that regular crystalline arrangements can take time to form with liposomes and annealing or extensive cold storage is required to produce these more stable cystalline arrangements. Even when the temperature is raised to 2°C the crystalline packing in the cell membrane is maintained and when the cells are irradiated, polymer chains are formed giving rise to the red-coloured membrane.

The loss of activity which arises as a result of polymerisation of the membrane indicates that the enzyme is dependent to some extent upon the physical state of the membrane lipids with which it is associated. De Kruijff et al. [16] did not observe a break in the Arrhenius plots of the NADH oxidase activity of *A. laidlawii* membranes despite the fact that the membrane lipids underwent a phase transition in the temperature range studied. They therefore concluded that the enzyme did not depend on the physical state of the membrane lipids for activity. Whether the loss of activity which we observe has to do directly with the cross-linking preventing conformational changes or movement of the enzyme or is simply due to a decrease in the mobility of substrates and products in this more rigid system, we do not yet known. Polymerisation only occurs when the lipids are in the crystalline state after storage at low temperature. Under these conditions the protein and lipid will undergo phase separation to form patches of aggregated protein [23]. Following irradiation these patches will not be able to disaggregate as the temperature is increased, and this may influence activity. The $(Ca^{2+} + Mg^{2+})$-ATPase from rabbit muscle sarcoplasmic reticulum when reconstituted into C_{20} diacetylenic phosphatidylcholine also shows a loss of activity as a result of polymerisation (Leaver and Restall, unpublished results). This enzyme is known to undergo rotational diffusion in the plane of the lipid bilayer matrix of this membrane.

The failure of polymerisation to affect the activity of the extrinsic membrane-bound enzyme, ribonuclease, was not unexpected. Wagner et al. [24] reported that polymerisation of diacetylenic-sulpholipid liposomes containing the membrane-bound ATP-synthetase complex from *Rhodospirillum rubrum* did not reduce the ATPase activity. This activity is also located in the extrinsic portion of the protein molecule. Since the extrinsic proteins are not as intimately associated with the membrane lipids as the intrinsic proteins, they are less likely to be inhibited by changes in their lipid environment.

We do not as yet know whether any cross-linking of the lipid to amino acids of the proteins occurs. If this were to occur it could yield information regarding the positional relationships of specific proteins and lipids in the membrane, i.e.

whether a particular protein is associated with a specific lipid class. This is currently under investigation using ATPase-diacetylene lipid complexes.

The effect of UV irradiation on the activity of the membrane NADH oxidase is interesting. Purohit et al. [25] showed that one effect of gamma irradiation on human erythrocyte membranes was to decrease the number of membrane sulphydryl groups. The stabilising effect of dithiothreitol, a much-used sulphydryl group-protecting agent, suggests that the inactivation of NADH oxidase observed in the absence of any sulphur-containing radioprotectants was due to loss of sulphydryl groups. Reinards et al. [17] in their studies of the purified NADH oxidase from *A. laidlawii* observed that enzymatic activity was strongly inhibited by *p*-chloromercuribenzoate and $HgCl_2$ suggesting that sulphide or sulphydryl-groups may be essential for activity. Inactivation by ultraviolet light radiation of this key enzyme may be the mechanism associated with the killing effects of radiation on bacterial cells. The enhanced inhibition of this enzyme as a result of polymerisation probably increases the killing effect of ultraviolet light radiation on cells containing C_{20} diacetylenic fatty acid. This is currently being investigated.

A further potential use which arises from the present studies is to use the *A. laidlawii* cells as a source of polymerisable lipids, particularly the glycolipids which are present in large amounts in the cell membrane. Stable surfaces coated with these lipids and then polymerised may find a use for studies involving cell-cell contact via the carbohydrate polar groups.

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