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Electron spin resonance studies of lipid fluidity changes in membranes of an uncoupler-resistant mutant of *Escherichia coli*

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The fluidity of the lipids in membrane preparations from a mutant of *Escherichia coli* resistant to the uncoupler CCCP, grown at different temperatures with and without CCCP, was examined by electron spin resonance using the spin probe 5-doxyl stearic acid. The fluidity of the membrane lipids at the growth temperature, as estimated using electron spin resonance, was less in cells grown at lower temperatures. Precise homeoviscous adaptation was not observed. Growth in the presence of CCCP resulted in a decrease in membrane lipid fluidity, particularly in the inner (cytoplasmic) membrane. There was no change in the proportion of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin in the cell envelope. However, there was an increase in the proportion of unsaturated fatty acids in membranes from cells grown with uncoupler. This was reflected in the increased fluidity of the lipids extracted from these membranes. This result is contrary to that expected from measurements of the fluidity of the lipid in these membranes. The decreased fluidity of the lipid in these membranes may be a consequence of the observed increase in the ratio of protein to phospholipid.

Introduction

According to the chemiosmotic hypothesis of Mitchell [1], membrane energization for ATP synthesis or solute transport involves the translocation of protons across the energy-transducing membrane. Uncouplers dissipate the energized membrane state and so prevent ATP formation and inhibit the transport of those solutes which are energized by it [1]. It has been proposed that uncouplers dissolve in the membrane lipid and act as mobile proton conductors to equilibrate the proton gradient [1–3]. However, there is some evidence that uncouplers act on membrane proteins [4–8]. In order to learn more of the mechanism of uncoupling, and consequently of the mechanism of energy transduction, we have studied the properties of mutants of *Escherichia coli* which are resistant to the uncouler CCCP. (An account of the isolation and properties of these mutants will be published elsewhere.) These mutants are able to grow on Penassay Broth in the presence of 250 μM CCCP, a concentration lethal for other strains. If the cells are harvested following growth in the presence of CCCP, it is evident from the intense yellow colour of the cell pellet that the CCCP, or a metabolite of it, is accumulated by the cells. The principle sites of accumulation are the outer and inner (cytoplasmic) membranes of the organism.

In the present paper, we have examined the

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

properties of the lipids in membranes from an uncoupler-resistant mutant grown at different temperatures with CCCP. The fluidity of the lipids was examined by electron spin resonance using the spin probe 5-doxyl stearic acid. Growth in the presence of CCCP resulted in a decrease in membrane lipid fluidity, particularly in the cytoplasmic membrane. There was no change in the proportion of phosphatidylethanolamine, phosphatidylglycerol or cardiolipin in the cell envelope. However, there was an increase in the proportion of unsaturated fatty acids in membranes from cells grown with uncoupler. This was reflected in the increased fluidity of the lipids extracted from these membranes. Thus, the decreased lipid fluidity in the membrane may be a consequence of the increase observed in the ratio of protein to phospholipid when the cells are grown with uncoupler. The decrease in membrane fluidity or alterations in membrane proteins may be responsible for protecting the uncoupler-resistant cells from the effects of the uncoupler.

Materials and Methods

Bacterial strains

E. coli AN180 (*thi argE mtl xyl rpsL*) is the parent of the CCCP-resistant strain UV6 used in this study. Some of the characteristics of UV6 are described in Ref. 9.

Growth of cells

AN180 and UV6 were grown at 37°C to the stationary phase from a 1% (v/v) inoculum on Penassay Broth (Difco) in static cultures. UV6 was grown initially in the presence or absence of 50 μM CCCP as required. This culture was used to give a 1% inoculum for larger scale growth at the required concentration of CCCP.

Isolation of membranes

Cells were washed twice by resedimentation from 10 mM Hepes-KOH (pH 7.4). The cells (about 5 g wet weight) were resuspended in 10 ml of this buffer. DNAse and RNAse at 40 μg/ml were added and the cells disrupted by passing twice through a French pressure cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 1000 $\times g$ for 20 min. Cell envelopes were prepared by centrifuging the supernatant at 160000 $\times g$ for 1.5 h in a Beckman 42.1 rotor. They were washed twice with Hepes buffer.

Isolated inner and outer membrane fractions were prepared as follows. 5.0 ml of the supernatant was applied to each tube of a Beckman SW27 rotor containing a discontinuous gradient composed of 2.02 M (10 ml), 1.44 M (12 ml) and 0.77 M (12 ml) sucrose and centrifuged at 25000 rpm for 18 h. The inner membrane fraction, collecting at the interface between 0.77 and 1.44 M sucrose, was diluted approx. 3-fold in 3 mM EDTA (pH 7.4) and pelleted by centrifugation at 160000 $\times g$ for 2 h. The membrane pellet was resuspended in Hepes buffer. The outer membrane fraction, collecting at the interface between 1.44 and 2.02 M sucrose, was dialyzed against several changes of Hepes buffer to remove sucrose. The dialyzed preparation was concentrated to 2–3 ml by ultrafiltration with an Amicon PM10 filter.

Analysis of membrane fractions

Protein was measured by the method of Lowry et al. [10]. Total phospholipid content was measured by the method of Osborn et al. [11]. After extraction into $CHCl_3/CH_3OH$ (2:1), the phospholipids were oxidized to CO_2, H_2O and phosphate by combustion with $Mg(NO_3)_2$, and the liberated inorganic phosphate was then determined [12]. 1 μmol of inorganic phosphate was taken as equivalent to 0.7 mg phospholipid [11].

Phospholipids were identified by chromatography on thin-layer plates of Silica Gel G (250 μm thickness) using as solvent $CHCl_3/CH_3OH/H_2O$ (25:10:1). The phospholipids were visualized with I_2 vapour.

The fatty acid composition of membranes was determined using the procedure of Kates [13]. The fatty acids were esterified with 14% (w/v) boron trifluoride in methanol (BDH) (Supelco Bulletin 721B 'Esterification'). The methyl esters were separated by gas-liquid chromatography on a column of 10% DEGS-PS on 80/100 Supelcoport in a Hewlett-Packard 7610A gas chromatograph.

CCCP was extracted from cell envelopes or isolated membrane fractions by the method described by Osborn et al. [11] for phospholipids. The extracted CCCP was resuspended in ethanol (for 'proton pulse' experiments) or in $CHCl_3$. The

concentration of CCCP was determined using the molar extinction coefficient of $2.37 \cdot 10^4$ at the absorption maximum at 378 nm as described by Heytler [14].

Proton uptake by intact cells ('proton pulse' experiments)

The cells from 50 ml of an exponential phase Penassay Broth culture of *E. coli* AN180 were washed twice by resedimentation from 50 mM potassium phosphate (pH 6.8). The cells were suspended at 2 mg protein/ml in 2 mM Tris-HCl (pH 6.8)/50 mM KCl.

Proton translocation in response to a proton pulse was measured with a combination pH electrode as described in Ref. 15. A full-scale deflection on the chart recorder was equivalent to 0.2 pH units. The assay system contained 0.2 ml cell suspension in 2.0 ml 2 mM Tris-HCl buffer (pH 6.8) (containing 50 mM KCl). 20 μl 10 mM HCl was added as the 'proton pulse' followed by indicated amount of CCCP in ethanol. The changes in the pH of the medium were recorded.

Electron spin resonance measurements

The spin probe 5-doxyl stearic acid (Syva Corp., Palo Alto, CA) was dissolved as a 50 mM solution in absolute ethanol. The probe was incorporated by first evaporating a suitable aliquot of the ethanol solution to dryness under reduced pressure to form a film on the vessel in which it was contained. The solvent was completely removed by evacuation for a further 30 min on a vacuum line. A dispersion of membranes in Hepes buffer was then added to the vessel containing the spin probe. The resultant mixture was vortexed for 5 min and then allowed to stand on ice for 30 min, by which time the probe was incorporated into the membrane. In all experiments the ratio of spin probe to lipid was always less than or equal to 1 : 100 based upon lipid analysis and the quantity of spin probe used in the incorporation procedure. The membrane suspension was drawn up into a 20 μl disposable capillary pipette, one end of which was then sealed thermally. The membranes were then gently centrifuged down to the sealed end.

The capillary pipettes (three) containing the sample were inserted into the rectangular mode TE_{120} cavity of an X-band ESR spectrometer and the spectra were digitally recorded at 9.05 GHz. The fully calibrated (magnetic field and microwave frequency) ESR spectra were analyzed using a digital acquisition system described elsewhere [16]. The computerized system permitted baseline flattening and accurate measurement of spectral features by fitting each peak individually to a quadratic function, hence leading to a very precise measurement of $2T_{11}^{1}$. The temperature was controlled using a Varian E-257 temperature controller and was measured using a fine wire copper-constantin thermocouple inserted in the cavity. The spectra at each temperature were recorded with either a 5 min or a 10 min scan over 0.01 T (100 G) using a time constant of 0.125 or 0.400 s on the Ithaco 391A lock-in amplifier. The power level on the sample was never greater than 10 mW and the amplitude of the 100 kHz field modulation was 0.5 G. The spectra were recorded over the temperature range 2–50°C and the rate of change of temperature was maintained at 8 Cdeg/h.

Results

Lipid composition of mutant membranes

E. coli UV6 was grown at different temperatures with and without the presence of 75 μM CCCP in the growth medium. Whereas the cells grew well between 28 and 40°C, growth was very poor both above and below these temperatures. This fact influenced the nature of our experiments. We were unable to accumulate sufficient material from cells grown below 28°C or above 40°C to be able to study outer and inner membranes independently. Consequently, envelope preparations containing both outer and inner membranes were used in many of the experiments. Adequate amounts of outer and inner membranes were obtained from cells grown at 37°C.

The relative amounts of phosphatidylethanolamine, phosphatidylglycerol and of cardiolipin in envelopes, outer and inner membranes of CCCP-resistant strain UV6 were the same when the cells were grown at 37°C with or without 75 μM CCCP. However, there were marked changes in the fatty acid composition of the membrane lipids as a result of differences in the growth temperature and the presence of the uncoupler (Table I). There was

TABLE I

FATTY ACID COMPOSITION OF CELL ENVELOPE LIPIDS OF STRAINS AN180 AND UV6 GROWN AT DIFFERENT TEMPERATURES WITH AND WITHOUT 75 μM CCCP

Values are means of four separate envelope preparations. The fatty acids are myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), *cis*-9,10-methylenehexadecanoic acid (Δ17), stearic (18:0), and *cis*-vaccenic acid (18:1).

	Temp. (°C)	CCCP	Fatty acid esters (% (w/w))						
			14:0	16:0	16:1	Δ 17	18:0	18:1	16:1+Δ17+18:1
AN180	37	–	5.3	48.2	14.4	19.3	0.9	12.0	45.7
UV6	19	–	4.1	36.6	34.6	0.9	1.9	21.9	57.8
		+	4.4	34.4	33.8	1.0	1.7	24.8	59.6
	24	–	4.7	32.4	32.0	8.2	0.6	22.3	62.5
		+	4.3	34.6	34.6	2.9	0.9	22.5	60.0
	37	–	6.0	47.5	11.6	20.8	0.4	11.9	44.3
		+	6.0	46.3	20.4	8.8	0.9	17.0	46.2
	42	–	6.5	50.2	11.4	21.6	0.9	9.2	42.2
		+	4.7	47.5	14.4	16.0	0.9	16.0	46.4

an increase in the relative amounts of palmitic (16:0) and *cis*-9,10-methylenehexadecanoic (Δ17) acids as the temperature of growth was increased. By contrast, there were smaller amounts of palmitoleic (16:1) and *cis*-vaccenic (18:1) acids. As a consequence, the ratio of unsaturated plus cyclopropane (Δ17) fatty acids to saturated fatty acids decreased with an increase in growth temperature. This has been observed previously [17–21] and interpreted as an adaptation by the cells to maintain a constant viscosity of the membrane lipids at all temperatures [21]. Growth in the presence of CCCP at most temperatures resulted in a decrease in the relative amount of *cis*-9,10-methylenehexadecanoic (Δ17) acids and increases in the levels of palmitoleic (16:1) and *cis*-vaccenic (18:1) acids (Table I). The same types of change were observed in the isolated membranes from cells grown at 37°C (Table II). The increase in the ratio of unsaturated plus cyclopropane (Δ17) fatty acids to saturated fatty acids produced by growth in the presence of CCCP, which is indicated by the data for envelopes (Table I), is more clearly seen in the isolated cytoplasmic (inner) membrane (Table II).

Envelopes from the parent strain grown at 37°C had a fatty acid composition similar to those from the mutant grown at 37°C without CCCP (Table I).

ESR studies of membrane lipid fluidity

The effects of growth at different temperatures in the presence and absence of CCCP on the

TABLE II

FATTY ACID COMPOSITION OF MEMBRANE LIPIDS OF UV6 GROWN AT 37°C WITH AND WITHOUT 75 μM CCCP

Values are means of three separate preparations of inner and outer membranes.

Membrane	CCCP	Fatty acid esters (% (w/w))						
		14:0	16:0	16:1	Δ 17	18:0	18:1	16:1+Δ17+18:1
Inner	–	3.8	41.9	12.5	29.0	0.7	12.2	53.7
	+	3.4	36.9	24.4	14.3	0.7	20.3	59.0
Outer	–	5.9	50.0	12.8	17.4	0.8	13.2	43.4
	+	4.0	50.5	14.0	11.8	1.1	18.4	44.2

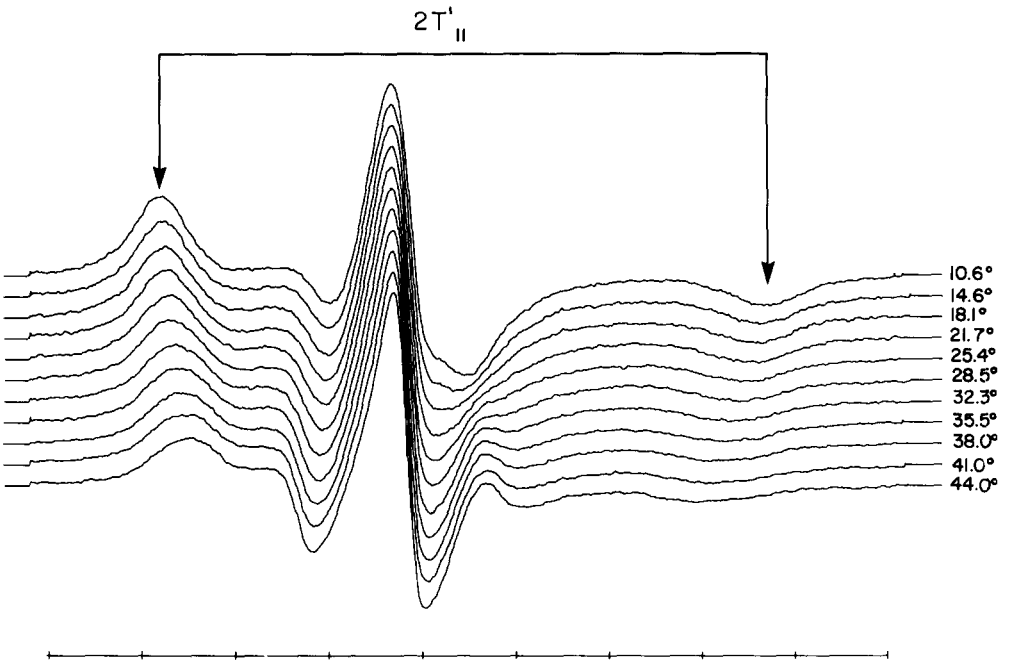

Fig. 1. ESR spectra of 5-doxyl stearic acid in envelopes of *E. coli* UV6 at various temperatures (°C). The envelopes were isolated from cells grown at 42°C without CCCP. The measurement of $2T_{11}^{1}$ is indicated. The field calibration starts at 3180 G and each increment is 10 G. The microwave frequency was 9.036 GHz with an incident power of 10 mW and a modulation amplitude of 0.36 G.

fluidity of the membrane lipids was examined by electron spin resonance using 5-doxyl stearic acid as the spin probe.

Spin labels are sensitive and informative monitors of molecular organization and dynamics in membranes. The perturbation caused by the nitroxide-containing ring is tolerable provided only relative changes (trends) in motion and organization in membranes are required. The probe 5-doxyl stearic acid is particularly useful in this regard, since its ESR spectrum provides a readily measurable parameter, $2T_{11}^{1}$, the splitting between the outer extrema observed in the spectrum, which is related to the motional order in the system [22]. The change in $2T_{11}^{1}$ from sample to sample and with temperature can be viewed as a measure of the mobility of the probe in the sample and hence as a reflection of changes in fluidity in the membrane. A large value of $2T_{11}^{1}$ is indicative of relatively low motional freedom of the probe and is interpreted as indicating that the probe is located in a membrane of low fluidity. The use and limitations of spin probes have been thoroughly discussed by us in Ref. 22 and by Schreier et al. [23].

Typical spectra of the probe 5-doxyl stearic acid in envelopes of *E. coli* UV6 at various temperatures are shown in Fig. 1. As the temperature increases, it is seen that the splitting $2T_{11}^{1}$ decreases, indicating a greater degree of motion of the probe. Outer extrema broaden at higher temperatures as expected from the work of Mason and Freed [24] and Meirovitch et al. [25]. Moreover, their work has shown that broadening should not be interpreted necessarily as indicating that there is more than one domain for the probe. Fig. 2 shows the variation of $2T_{11}^{1}$ with temperature for envelopes from cells grown at 19, 24, 37 and 42°C with and without 75 μM CCCP. There are no obvious discontinuities in the $2T_{11}^{1}$ versus temperature curves, suggesting that there were no measurable sharp phase transitions over the temperature range of the observations. This is as expected for such a heterogeneous system. Inspection of the data shown in Fig. 2 shows that as the growth temperature was increased, the value of $2T_{11}^{1}$ at any given temperature increased, indicating that the fluidity of the membrane lipid had decreased. The same trend was observed for cells grown with

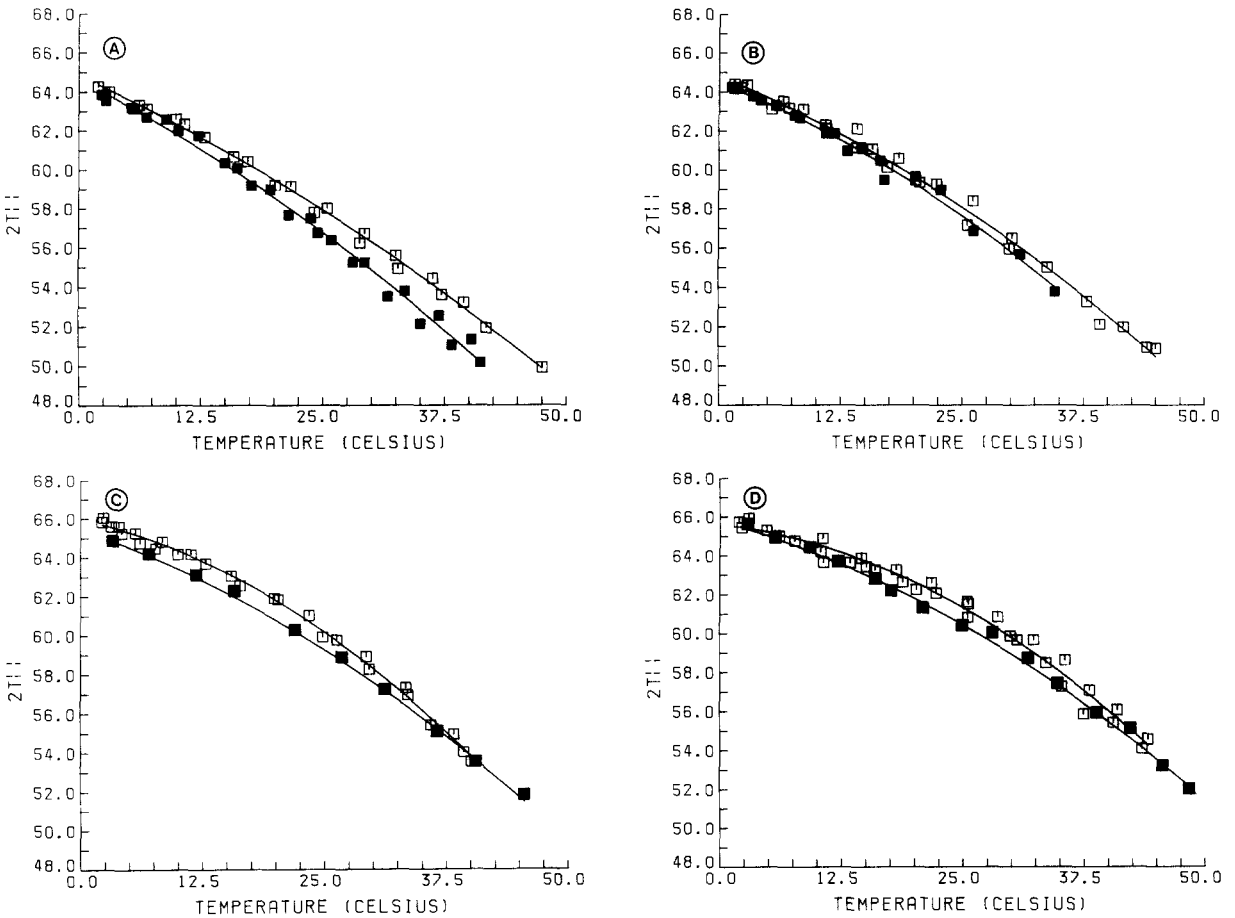

Fig. 2. The temperature variation of $2T_{11}^{1}$ for 5-doxyl stearic acid in envelopes of *E. coli* UV6 grown at various temperatures in the absence (closed points) and presence (open points) of 75 μM CCCP. A, 19°C; B, 24°C; C, 37°C; D, 42°C.

CCCP. Growth of the organism at a particular temperature in the presence of CCCP, led to a value of $2T_{11}^{1}$ that was slightly greater than that measured for membranes obtained from envelopes grown in the absence of CCCP. This effect was seen most clearly when the separated outer and inner membranes from cells grown at 37°C were examined (Fig. 3). The fluidity of the lipids in the outer membrane was less than that of the lipids in the inner membrane and showed no response to the presence of CCCP during growth. By contrast, the fluidity of lipids in the inner membrane from cells grown with CCCP was much reduced compared to that in the inner membrane from cells grown without CCCP. We did not repeat these experiments with outer and inner membranes from cells grown at other temperatures since, as described above, it was not possible to obtain sufficient outer and inner membranes for these experiments.

The lipids in envelopes from cells grown at 37°C with and without 75 μM CCCP were extracted into chloroform/methanol (2 : 1). The solvent was removed by evaporation under reduced pressure, the lipids were taken up in Freon II [26] and then dispersed in Hepes buffer. The Freon was completely removed by evacuation on a vacuum line. Following addition of the spin probe, the electron spin resonance measurements were carried out as before. Fig. 4 shows the variation of $2T_{11}^{1}$ with temperature. At all temperatures the value of $2T_{11}^{1}$ for lipid extracted from envelopes

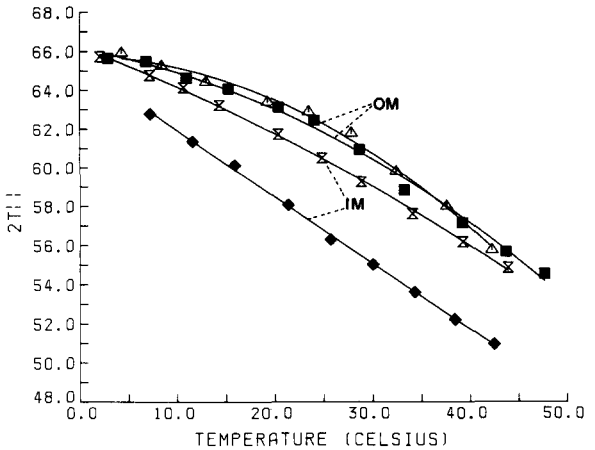

Fig. 3. Temperature variation of $2T_{11}^{1}$ for 5-doxyl stearic acid in outer (OM) and inner membranes (IM) of *E. coli* UV6 grown with (OM, closed points; IM, open points) or without (OM, open points; IM, closed points) 75 μM CCCP.

from cells grown with CCCP was lower than that from cells grown without the uncoupler. Thus, the increased fluidity of the lipids from cells grown with CCCP reflects the increased proportion of unsaturated plus cyclopropane fatty acids present.

Incorporation of CCCP into cell membranes during growth

Envelopes from cells grown with CCCP were markedly yellow, suggesting that CCCP, or a metabolite, had been incorporated. Extraction of the envelopes with chloroform/methanol (2 : 1) gave a yellow solution which had an absorption spectrum identical to that of CCCP. The yellow

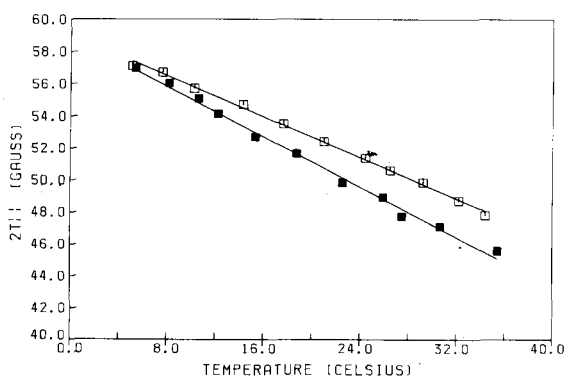

Fig. 4. Temperature variation of $2T_{11}^{1}$ for 5-doxyl stearic acid in lipids extracted from envelopes of cells grown at 37°C with (closed points) and without (open points) 75 μM CCCP.

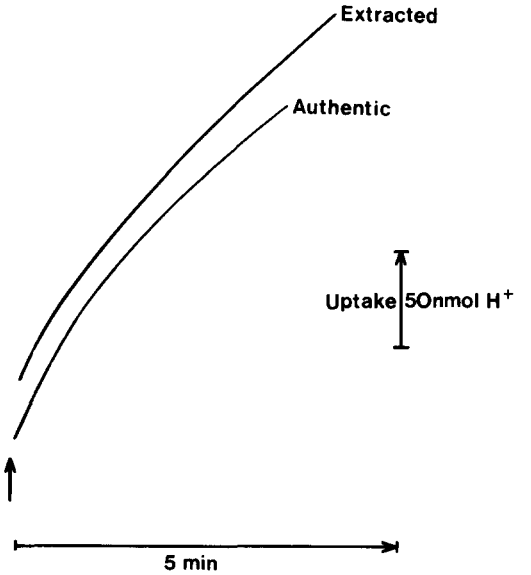

Fig. 5. Uptake of protons by a cell suspension of *E. coli* AN180 induced by the addition of authentic CCCP or of CCCP extracted from cell envelopes of UV6 which had been grown at 37°C with 75 μM CCCP. The experiment was carried out as described in Materials and Methods. The pH change due to the prior addition of HCl is not shown. CCCP (5 μl in ethanol) was added at the arrow to give a final concentration of 12.5 μM.

material co-chromatographed with authentic CCCP on thin-layer plates of Silica Gel G using $CHCl_3/CH_3OH/H_2O$ (25 : 10 : 1) as solvent. The amount of CCCP in the solvent extract, and thus in the membrane lipids, was measured using the extinction coefficient given by Heytler [14]. A comparison of the effectiveness of CCCP extracted

TABLE III

PHOSPHOLIPID AND CCCP CONTENTS OF OUTER AND INNER MEMBRANES OF UV6 GROWN AT 37°C WITH AND WITHOUT 75 μM CCCP

Values are means of three separate preparations of outer and inner membranes.

Membrane	CCCP (during growth)	Concentration in membrane	
		CCCP (mol/mol phospholipid)	Phospholipid (mg/mg protein)
Outer	–	–	0.09 ± 0.02
	+	0.25 ± 0.24	0.06 ± 0.02
Inner	–	–	0.16 ± 0.02
	+	0.081 ± 0.033	0.08 ± 0.02

from membranes with authentic CCCP in equilibrating a proton gradient across the envelope of intact cells of wild-type *E. coli* is shown in Fig. 5. In this experiment cells were suspended in a weakly buffered solution. When the pH of the suspension had become stable, a 'pulse' of dilute HCl sufficient to lower the pH by 0.08 pH units was added. Protons were slowly taken up by the cells. The rate of uptake was greatly accelerated by the addition of authentic CCCP or by an equivalent concentration of CCCP extracted from membranes. Thus, the material in the membrane lipid still retained the proton-equilibrating properties characteristic of many uncouplers [1–3].

The phospholipid and CCCP contents of inner and outer membranes from cells grown at 37°C with and without CCCP are shown in Table III. CCCP was found in both membranes with more being present in the outer membrane. There was considerable variability in the amount present. Some CCCP had also reacted with protein in the membranes. It was not extracted by $CHCl_3$/CH_3OH unless 1% (w/v) dithiothreitol was present. The amount bound in this form was variable, usually being about one-third of that present in the lipid fraction.

Table III also shows that growth in the presence of CCCP decreased the phospholipid : protein ratio of the inner and outer membranes.

Discussion

Two main points emerge from the results described in this paper. First, the fluidity of the membrane lipids of the CCCP-resistant strain *E. coli* UV6 is not the same at different growth temperatures. Second, growth of this strain in the presence of CCCP results in incorporation of the uncoupler into the lipid domain of the inner and outer membranes; the fluidity of the lipids in these membranes is reduced.

Marr and Ingraham [17] first observed that *E. coli* adjusts the ratio of unsaturated to saturated fatty acids in its membrane phospholipids in response to growth temperature. As the temperature of growth is decreased the proportion of *cis*-vaccenic acid is increased. Sinensky [21] proposed that the variation of the fatty acid composition of the membrane phospholipids serves to produce membranes whose lipids have a constant fluidity at the temperature of growth. This process was termed 'homeoviscous adaptation'. This concept has been supported by studies of *Bacillus stearothermophilus* and an oral strain of *Acholeplasma laidlawii* [27,28]. By contrast, other workers have found that lipid fluidity can vary appreciably without impairing essential membrane functions [29–32]. McElhaney and co-workers [29] have suggested that in fact 'homeoviscous adaptation' does not maintain absolutely constant membrane fluidity but is a means of adjusting the lipid composition in order to maintain a proper lipid phase state such as liquid crystalline or a mixture of gel and liquid crystalline.

The results of the present study add support to the perception that 'homeoviscous adaptation' is not a precise control of membrane fluidity at a critical value for cell growth. As the growth temperature of UV6 was increased, the ratio of unsaturated plus cyclopropane ($\Delta 17$) fatty acids to saturated fatty acids decreased, leading to relatively more rigid membranes at a particular temperature of observation; there is in fact a 4 G difference in $2T_{11}^{1}$ between the samples grown at 19°C and 42°C. An interesting way of further analyzing these data is to plot the values of $2T_{11}^{1}$ against the temperature of observation of the spectrum minus the growth temperature ($T_{obs} - T_g$). In this plot the fluidity of the membrane lipids at the growth temperature, as indicated by $2T_{11}^{1}$ at 0°C, can be compared directly. The plots for cells grown at the various growth temperatures with and without CCCP are shown in Fig. 6.

The plots of $2T_{11}^{1}$ against $T_{obs} - T_g$ are parallel for the four growth temperatures, demonstrating clearly the lack of precise homeoviscous adaptation (if it were present the lines would be expected to intersect). Furthermore, the graphs, and the values summarized in Table IV, show that the fluidity of the membrane lipid at the growth temperature in cells grown at 19°C was lower than in cells grown at 42°C, despite the fact that the membranes for those cells grown at the lower temperature contained more unsaturated lipids. Clearly, the increase in unsaturated fatty acids was insufficient to completely offset the lower growth temperature. However, these results suggest that there is a range of fluidity of the membranes over

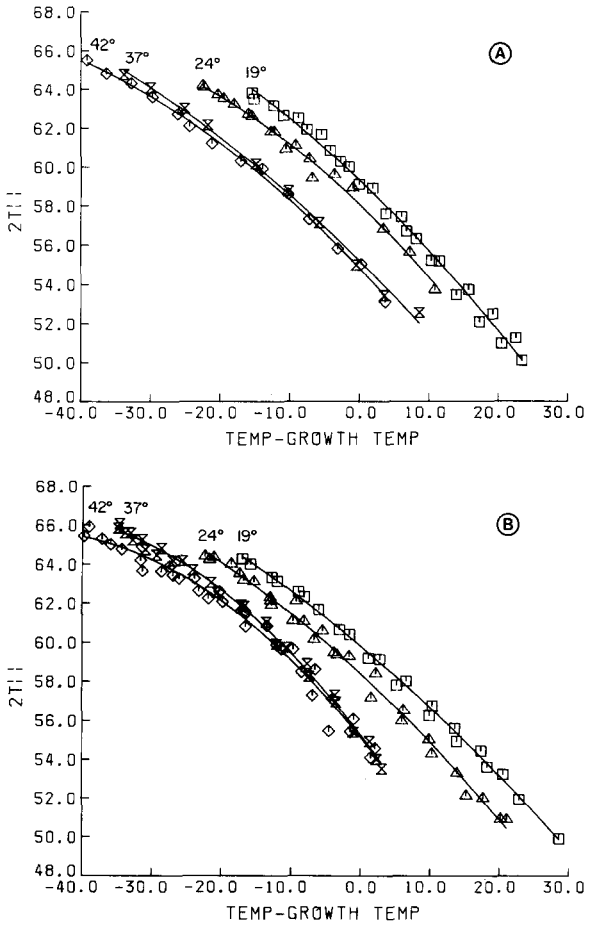

Fig. 6. Variation of $2T_{11}^{1}$ for 5-doxyl stearic acid in envelopes of *E. coli* UV6 as a function of the difference between the temperature of measurement and the growth temperature. The envelopes were isolated from cells grown at various temperatures with (B) or without (A) 75 μM CCCP.

which the cells are viable. Similar results were obtained for cells grown in the presence of CCCP.

Growth of *E.* coli UV6 occurs normally in the presence of concentrations of CCCP which are lethal for wild-type strains. The mechanism of resistance has not been elucidated. It does not appear to be due to decreased permeation of the uncoupler. Various bioenergetic properties are altered in strain UV6 [9]. As described in the present paper, growth of UV6 in the presence of CCCP results in the incorporation of the uncoupler into the lipid domains of both the inner and outer membranes. (Although the material incorporated into the membrane lipid has not yet been rigorously characterized as unmodified CCCP, it has identical light absorption and chromatographic properties, and is as effective an uncoupler as authentic CCCP.) The fluidity of the lipids, particularly in the inner membrane, is decreased in membranes grown with CCCP, although the fluidity of the lipids extracted from these membranes is greater than that of the lipids from cells grown without the uncoupler. This is in accord with the increased proportion of unsaturated and cyclopropane fatty acids in the lipids from cells grown with CCCP. The anomalous fluidity of the lipids in the membrane is probably not due to a rigidifying effect of CCCP, since most of the uncoupler is extracted with the lipids from the membrane. It did not rigidify in the experiments with the extracted lipids. The decrease in fluidity of the lipid in the membranes of cells grown with CCCP is most likely due to the effect of protein. We have observed that there is an increased protein : phospholipid ratio in these membranes. The nature of this protein is currently being investigated.

Dombek and Ingram [33] have recently reported on the effect of growing *E. coli* at high concentrations of ethanol. Their results show a remarkable parallel with those reported here. The lipids in inner membranes from cells grown with ethanol were less fluid, in spite of the increase in the proportion of *cis*-vaccenic acid esterified in the phospholipids. There was also a decrease in the lipid-to-protein ratio. However, Dombek and Ingram did not report on whether ethanol had been incorporated into the lipid phase of the membranes.

TABLE IV

FLUIDITY AT THE GROWTH TEMPERATURE OF ENVELOPE LIPIDS OF UV6 GROWN WITH AND WITHOUT 75 μM CCCP

Lipid fluidity as measured by $2T_{11}^{1}$ was obtained from the data in Fig. 5.

Growth temperature (°C)	$2T_{11}^{1}$ at growth temperature (G)	
	−CCCP	+CCCP
19	59.2	59.8
24	58.0	58.4
37	55.1	55.4
42	54.8	55.0

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