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Biochemical and morphological properties of membranes of unsaturated fatty acid auxotrophs of *Salmonella typhimurium*: effects of fluorinated myristic acids

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In order to investigate the utility of the fluorine-19 nucleus as a spectroscopic probe, a fluorinated analog of myristic acid has been incorporated into the membrane lipids of an unsaturated fatty acid auxotroph of *Salmonella typhimurium*. It is capable of supporting limited growth at temperatures above 37°C. Freeze-fracture electron microscopic examinations of the membrane ultrastructure show a temperature and fatty acid supplement-dependent segregation of intramembranous protein particles into distinct patches in the auxotrophic membrane leaving intramembranous protein-denuded areas. The occurrence of these patches seems to be related to the phase separation of membrane lipids. Corresponding changes in the transport and accumulation of methyl thio-β-D-galactopyranoside and tetracycline are observed. However, transport of histidine does not appear to be dependent on the physical state of the membrane lipids. The auxotroph shows differences in growth and morphological characteristics from those of the wild type. Functions of both inner and outer membranes are shown to be affected as a response to the fatty acid chain composition of the lipids.

Introduction

The physical state of membrane lipids is believed to have significant influence on the functions of the membranes. Many investigators, applying a number of techniques, have examined the effects of lipids and lipid structure on physical, morphological, and functional characteristics of biological membranes [1–10]. Fatty acid auxotrophs of Gram-negative bacteria provide a good system to study these effects, since the acyl chain composition in the membrane lipids of these organisms can be controlled by the fatty acid provided in the external growth medium. Unsaturated fatty acid auxotrophs of *Escherichia coli* have been particularly useful for investigations relating structural changes to alterations in functional properties. Changes in such diverse physiological activities as chemotaxis, DNA synthesis, and transport have been attributed to differences in membrane fluidity reflecting altered fatty acid composition and physical state of the membrane lipids [11–16].

Of interest to us has been the application of NMR spectroscopy to the study of structure-function relationships and protein-lipid interactions

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taking place in biological membranes. Because of the many advantages offered by the ^{19}F nucleus as a probe (for review, see Ref. 17), ^{19}F-NMR has been of particular concern. Tryptophan auxotrophs of *E. coli* and *Salmonella typhimurium* have been useful for labeling membrane proteins and periplasmic proteins with fluorinated analogs of tryptophan [18–20]. The availability of fatty acid auxotrophs of *E. coli* has made it possible for us to label membrane lipids of these organisms with a fluorinated analog of myristic acid in quantities sufficient to enable us to examine biological membrane lipids by means of ^{19}F-NMR [21]. Using this technique, we have reported evidence suggesting differences in protein-lipid interactions between unsaturated fatty acid auxotrophs derived from two strains of *E. coli* (K and ML) [22]. In order to extend these studies and evaluate possible perturbing effects of the ^{19}F probe in systems other than *E. coli*, we have investigated some of the membrane-associated characteristics of unsaturated fatty acid auxotrophs of the closely related organism *S. typhimurium* under conditions which alter the fatty acid composition of the membrane lipids. In addition, by means of freeze-fracture electron microscopy (EM), we have examined the morphological fine structure characteristics of the cell membranes subsequent to temperature changes and incorporation of 8,8-$^{19}F_2$-labeled myristic acid (8,8-$^{19}F_2$ 14 : 0).

Materials and Methods

Media, bacterial strains, and growth

Nutrient broth (Difco) with 5 g NaCl per liter, or L Broth (23) was used as rich medium. Proteose peptone No. 3 beef extract agar consists of 0.1% beef extract, 1% Difco proteose peptone No. 3, and 0.5% NaCl [24]. Minimal medium consisted of 60 mM potassium phosphate buffer (pH 7.0), 0.01% $MgSO_4 \cdot 7H_2O$ and 0.1% $(NH_4)_2SO_4$. Carbon sources were sterilized separately and added to a final concentration of 0.2 to 0.5%. Amino acids were used at a concentration of 20 μg/ml, uracil at 5 μg/ml. Fatty acids were brought to pH 7.0 with potassium hydroxide and stored at −20°C as 10% solutions in 80% ethanol. When required, oleic (18 : 1), palmitoleic (16 : 1), or myristoleic (14 : 1) acids were added to final concentrations of 100 μg/ml and 8,8-$^{19}F_2$ 14 : 0 to 25 μg/ml. Brij 58 was used at 250 μg/ml, tetracycline hydrochloride at 25 μg/ml and for solid media, 1.5% agar was included. Growth rates were determined on a Bausch and Lomb 'Spectronic 20'. On our instrument, an absorbance of 0.1 at 570 nm (pathlength of 1 cm) is equal to 50 μg dry weight per ml of culture.

The strains used are all derivatives of *S. typhimurium* and are listed in Table I. TA1859 *dhuA*$_1$ was the gift of Dr. G.F.-L. Ames. It produces increased quantities of the periplasmic histidine-binding protein J (J protein). NK337, NK1017, and bacteriophage P22HTint3 were donated by Dr. David Botstein. CM5003 is an unsaturated fatty acid auxotroph derived by mutagenesis of TA1859 with nitrosoguanidine. It maps at 47 units on the *Salmonella* chromosome (Cottam, P.F. and Ho, C., unpublished results). Tn10 insertions within the histidine region of TA1859 were recovered by transduction of the Tn10 element from a pool of tetracycline resistant clones of *S. typhimurium* LT2 harvested from green indicator plates [25] following infection with P22Tc10 released from NK337 after temperature shift from

TABLE I

LIST OF STRAINS USED IN THIS WORK

Strain	Genotype or description	Source
LT_2	*wild type*	this laboratory
TA1859	*dhuA*$_1$	Ames, G.F.-L.
NK337	*leu* am414 *su*19 (P22Tc10)	Botstein, D.
NK1017	*pyr*C7 *str*^{r}/F'*ts*114*lac*$^+$ *zz*f535::Tn10	Botstein, D.
CM5003	*dhuA*$_1$ *ufa*	this laboratory
CM2200	*dhuA*$_1$*his*::Tn10/F'*ts*$^+$114 lac$^+$ *zzf*::Tn10	this laboratory
CM5203	*dhuA*$_1$ *ufa his*::Tn10/F'*ts*114 *lac*$^+$ zzf::Tn10	this laboratory

28 to 38°C [26]. A lysate of P22HTint3 grown on TA1859 *his*::Tn10 was then used to transduce CM5003 to Tet resistance. Histidine auxotrophs were tested for the ability to use D-histidine, a characteristic of the *dhuA* mutation.

Genes for the utilization of lactose were put into the histidine auxotrophs of TA1859 and CM5003 by means of the F'*ts*114 lac$^+$*zzf*::Tn10 plasmid harbored in NK1017 essentially as described by Chumley et al. [27]. Survivors CM2200 and CM5203 were obtained in this manner.

For the incorporation of 8,8-$^{19}F_2$14 : 0, cultures were grown to exponential phase at the specified temperature in media supplemented with 18 : 1 or 16 : 1. They were harvested by centrifugation at 4°C, and washed two times with 60 mM potassium phosphate buffer (pH 7.0). The cultures were divided, one portion resuspended in medium containing the original fatty acid supplement and the other supplemented with 8,8-$^{19}F_2$14 : 0 equilibrated to the proper temperature.

Membrane preparation, lipid extraction, fatty acid analysis, protein and phosphate determinations

Membranes were prepared by osmotic lysis as described by Osborn and Munson [28]. The inner and outer membranes were separated in a 2.2 ml 30–50% sucrose step gradient with an 0.5 ml 55% cushion using the SW40 rotor at 36000 rpm. Visible bands were collected from the top and the fractions from separate tubes pooled and washed.

Lipids were extracted from membranes or whole cells by the Bligh and Dyer procedure as modified by Ames [29]. Fatty acids were released and esterified in 2% H_2SO_4 in methanol at 70–80°C for 90 min [30]. The methyl esters were analyzed by gas chromatography on a 6 ft 10% DEGS-PS glass column (Supelco, Inc., Bellefonte, PA).

Proteins were measured by the Lowry procedure [31] with minor modifications. Lipid phosphates were determined using the method of Bartlett as modified by Bottcher et al. [32].

Freeze-fracture electron microscopy

Bacterial samples were grown, washed as described above, and rapidly frozen from given temperatures without cryoprotectant. Prior to freeze quenching, bulk samples were equilibrated at either 20 or 37°C for at least 30 min. 0.1 μl sample was placed between two thin copper plates kept at the respective temperatures. The sandwiched samples were further equilibrated at that temperature for three more minutes, then plunged into liquid propane using a rapid freezing plunger. The frozen samples were fractured and replicated at −120°C in a Polaron 7500 unit. The replicas were cleaned in nitric acid and Clorox, and examined in a Hitachi H-600 electron microscope.

Induction of β-galactosidase, assays and transport experiments

Cell cultures were grown to exponential phase in nutrient broth or minimal media with the appropriate supplements. They were divided, centrifuged and washed with the same medium lacking fatty acids and resuspended in fresh medium containing the original fatty acid, 8,8-$^{19}F_2$14 : 0, or nothing extra. Cultures were equilibrated 15 min before induction with 1.0 mM methyl thio-β-D-galactopyranoside or 0.6 mM isopropyl β-thiogalactopyranoside. β-Galactosidase activity was determined by sampling cultures periodically during growth. 5-ml samples were centrifuged of filtered for assays of the supernatants. Two 1-ml samples were treated with a drop of toluene, *ortho*-nitrophenyl galactopyranoside was added and the reaction was stopped by the addition of 0.5 ml of 1.0 M K_2CO_3. A blank was prepared by adding K_2CO_3 to the second tube before the addition of *ortho*-nitrophenyl galactopyranoside. Assays were read at 420 nm and the release of nitrophenol was determined using a millimolar extinction coefficient of 4.32.

Transport measurements were made using the rapid filtration technique on intact cells. Exponential phase cells, grown as required for the particular assay, were washed and resuspended in phosphate buffer containing 0.01% $MgSO_4 \cdot 7H_2O$. Chloramphenicol was added to 25 μg/ml. Cultures were kept on an ice bath until the start of the transport experiment, usually about 10 min. The experiment was started by adding a measured amount of prewarmed cells to prewarmed radioactive substrate in a small flask which was kept agitating slowly throughout the experiment. 1-ml samples were removed, filtered, washed with 5 ml of buffer, dried, and counted. Efflux of radioactive methyl thio-β-D-galactopyranoside was dem-

onstrated by adding a portion of the cells to a second flask containing 20 mM lactose after they reached steady state and then alternately sampling from both flasks.

Excretion of periplasmic proteins and sensitivities to external agents

Anti-J protein was produced in two female albino rabbits. 1 ml of purified J protein containing 2 mg/ml was mixed with equal parts of Freund's complete adjuvant. 2 ml were injected subcutaneously into the backs of the animals followed by two boosters given at 3-weekly intervals. Blood was collected by cardiac puncture. The serum was stored at −80°C subsequent to inactivation of antibacterial activity at 56°C for 30 min. It gave a single precipitin band with purified J protein. Tests for leakage of the periplasmic J protein were performed on fully supplemented agar plates. Anti-J protein was placed in a center well. The test organisms were inoculated into surrounding wells containing enough medium to permit growth of the organisms. The appearance of a precipitin band corresponding to that of purified J protein was used to indicate leakage of J protein from the cells into the external medium.

Leakage of RNAase was done on PPBE-18 : 1, -16 : 1 and -14 : 1 plates as described by Weigand and Rothfield [24]. Sensitivities to the inhibitory agents employed were determined by monitoring growth following addition of various concentrations to cultures of exponentially growing cells or by the reduction of colony-forming units on agar plates containing varying agent concentrations.

Chemicals

Fatty acids (18 : 1, 16 : 1 and 14 : 1) were obtained from Nuchek Prep (Elysian, MN) and 8,8-$^{19}F_2$14 : 0 was synthesized by Dr. Susan R. Dowd of this laboratory [21]. Chloramphenicol, tetracycline hydrochloride, methyl thio-β-D-galactopyranoside, *o*-nitrophenyl galactopyranoside and *Torula* RNA were obtained from Calbiochem Behring Corporation. Ethidium bromide and isopropyl β-thiogalactopyranoside were purchased from Sigma. Radioactive chemicals used were all products of New England Nuclear.

Results

Phase-contrast microscopic observations of the cell cultures reveal large variations in size and shape of the auxotrophic cells not seen in cultures of the parent strain grown under identical conditions (results not shown). The cellular morphology of CM5003 resembles that of some other mutant classes of *E. coli* and *S. typhimurium* which have been characterized as division defects, failure to initiate septum formation [24,33–35], or of the outer membrane to invaginate [36].

When 18 : 1 or 16 : 1 is provided as an unsaturated fatty acid supplement, growth rates are slightly reduced from those of the parent strain. These two supplements serve equally well in maintaining auxotrophic growth throughout the entire period at any given temperature until the culture reaches stationary phase. When the supplement provided is 14 : 1, growth rates are reduced 25% relative to those obtained with 18 : 1 or 16 : 1. Growth yields are the same and equal to those of the wild type provided the supply of supplement is adequate. The pattern of growth at 38 and 20°C is shown in Fig. 1A and B, respectively. Unlike 18 : 1 and 16 : 1, the unsaturate 14 : 1 is not a major component of *S. typhimurium* lipids (Table II below shows that in unsupplemented cultures of CM2200, 16 : 1, 18 : 1 (and the cyclopropane fatty acids derived from them) are the unsaturates synthesized by *S. typhimurium*). 14 : 1 does not play an important role in wild-type physiology. Thus, the use of this supplement does not appear to be relevant in assessing possible perturbing effects of 8,8-$^{19}F_2$14 : 0 in membrane lipids. Therefore, we have not included the results in Fig. 1A and B. Lacking any supplement, culture turbidities increase for approximately 1.25 doublings. At temperatures above 37°C, the analog 8,8-$^{19}F_2$14 : 0 supports an increase of more than two doublings. At lower temperatures or grown in the presence of myristic acid (14 : 0) (results not shown), the increase in the culture turbidity is less than when no fatty acid is provided. Colony forming units in supplemented cultures of the auxotroph are approx. 66% those of the wild type at the same absorbance; reflecting the morphological aberrations of the auxotroph noted above.

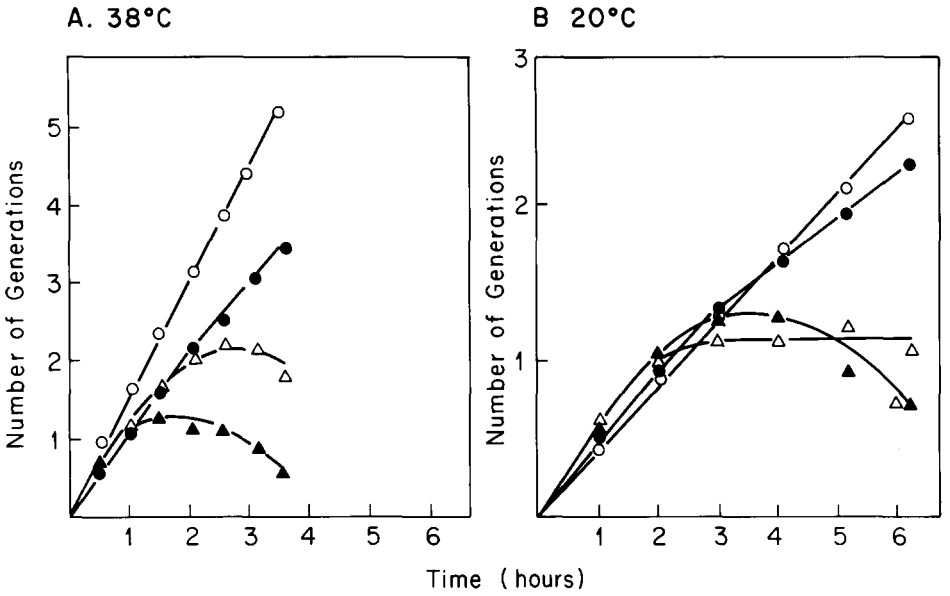

Fig. 1. Growth of *S. typhimurium* TA1859 and CM5003 in nutrient broth as a function of fatty acids and temperature: (A) 38°C and (B) 20°C. ○, TA1859 in 16:1, 18:1, or 8,8-$^{19}F_2$-14:0; ●, CM5003 in 16:1 or 18:1; △, CM5003 transferred to 8,8-$^{19}F_2$14:0; ▲, CM5003 with no additions.

Fatty acid analysis

Fatty acid analysis of the lipids of CM5203 and CM2200 are given in Table II. Cyclopropane fatty acids derived from 16:1 and 18:1 are included in the calculation of the methyl esters of those components. Not included in the calculations are small quantities of methyl laurate, two minor components with retention times between those of methyl myristate and methyl palmitate, methyl β-hydroxymyristate and trace amounts of methyl stearate. The data in Table II are from exponentially growing cells which have increased 0.8 doublings after being transferred to fresh media. CM2200 will incorporate exogenous 18:1 and 16:1, as is apparent by the increase in these components when they are present in the medium. It incorporates approx. twice as much 8,8-$^{19}F_2$ 14:0 at 20°C as it does at 30 or 40°C, but the ratio of unsaturated to saturated fatty acids at 20°C (where the demand for unsaturates is substantially greater), is still higher in the presence of 8,8-$^{19}F_2$14:0 than the ratio at higher temperatures of unsaturated fatty acid supplemented cells of CM5203. In unsupplemented cultures of CM2200, the quantity of 16:1 remains relatively constant, while the proportion of the other major unsaturate (18:1) increases with decreasing temperature.

The auxotroph incorporates 8,8-$^{19}F_2$14:0 at all growth temperatures although, as seen above, the analog will not support an increase in absorbance at lower temperatures where more 18:1 is normally synthesized by the parent strain rather than the saturated fatty acid found at higher temperatures. This demonstrates clearly that 8,8-$^{19}F_1$14:0 is not toxic to the cells. The increase in absorbance at higher temperatures is possible because the unsaturated fatty acid requirement is not so great. At lower temperatures, 8,8-$^{19}F_2$14:0 replaces 18:1 but does not satisfy the increased requirement.

Composition of membrane vesicles

With sucrose density centrifugation the membranes separate into four distinct bands L_1, L_2, M and H according to their buoyant densities [28,37]. Analysis of their phospholipids reveals that the fatty acid composition is nearly identical in each fraction (for details, see Table III). The only obvious difference between the four fractions is the small recovery of fraction L_1 from auxotrophic membranes containing 8,8-$^{19}F_2$14:0. This is reproducible, but whether it represents a degradation of

TABLE II

FATTY ACID COMPOSITION OF *S. TYPHIMURIUM* LIPIDS

Temperature (°C)	Strain	Supplement	Fatty acid composition (mol%)				
			14:0	16:0	16:1	18:1	8,8-$^{19}F_2$14:0
20	CM2200	none	6.0	29.1	30.8	34.0	–
		18:1	5.5	26.3	14.1	53.9	–
		18:1 → 8,8-$^{19}F_2$14:0	3.3	31.0	24.5	32.0	8.9
		16:1	5.1	33.0	41.2	20.4	–
		16:1 → 8,9-$^{19}F_2$14:0	4.6	30.0	30.4	23.6	11.0
	CM5203	18:1	2.8	34.5	6.1	56.4	–
		18:1 → 8,8-$^{19}F_2$14:0	17.4	44.2	5.9	18.2	14.0
		16:1	7.9	42.5	46.2	2.7	–
		16:1 → 8,8-$^{19}F_2$14:0	12.4	48.0	21.4	2.6	15.0
30	CM2200	none	11.6	33.3	31.9	22.9	–
		18:1	13.9	32.4	18.5	35.0	–
		18:1 → 8,8-$^{19}F_2$14:0	13.1	32.2	26.5	22.8	5.4
		16:1	14.5	33.1	36.1	15.8	–
		16:1 → 8,8-$^{19}F_2$14:0	5.0	34.3	32.6	21.6	5.7
	CM5203	18:1	20.5	36.3	9.7	33.0	–
		18:1 → 8,8-$^{19}F_2$14:0	19.4	41.5	9.2	17.4	12.4
		16:1	20.4	40.1	36.4	2.4	–
		16:1 → 8,8-$^{19}F_2$14:0	16.0	48.0	21.3	2.4	12.5
40	CM2200	none	11.9	39.8	31.3	16.6	–
		18:1	14.4	33.3	18.4	33.0	–
		18:1 → 8,8-$^{19}F_2$14:0	14.0	33.0	25.5	23.0	4.2
		16:1	16.7	34.2	36.8	12.2	–
		16:1 → 8,8-$^{19}F_2$14:0	16.5	33.7	29.4	15.4	4.7
	CM5203	18:1	16.8	36.4	7.7	39.0	–
		18:1 → 8,8-$^{19}F_2$14:0	17.7	39.8	7.7	17.6	16.4
		16:1	12.1	42.2	44.0	1.6	–
		16:1 → 8,8-$^{19}F_2$14:0	21.1	44.2	17.0	1.6	16.0

TABLE III

DISTRIBUTION OF PROTEINS AND PHOSPHOLIPIDS IN MEMBRANES AND MEMBRANE FRACTIONS OF CM5003 AND TA1859 (t.m., total membrane)

Strain	Fraction	Weight (mg/ml)		Percent of total recovered		Ratio protein/lipid
		lipid	protein	lipid	protein	
TA1859	t.m.	3.63	5.60			1.55
	L_1	0.89	0.23	31.4	5.1	0.26
	L_2	0.83	1.25	29.3	28.0	1.50
	M	0.65	0.80	23.0	17.7	1.23
	H	0.46	2.18	16.2	48.8	4.70
CM5003	t.m.	6.49	10.20			1.57
(18:1)	L_1	2.24	0.73	25.0	5.1	0.32
	L_2	2.69	3.84	30.0	26.9	1.42
	M	1.98	2.30	22.1	16.1	1.16
	H	2.05	7.40	22.8	51.9	3.60
CM5003	t.m.	5.27	10.80			2.00
(8,8-$F_2$14:0)	L_1	1.14	0.34	10.7	1.8	0.30
	L_2	3.55	4.80	33.3	25.6	1.35
	M	2.14	3.10	20.1	16.5	1.45
	H	3.82	10.50	35.9	56.0	2.74

L_1 due to the depletion of unsaturated fatty acids or the incorporation of 8,8-$^{19}F_2$14 : 0 is not clear . L_1 and L_2 contain the cytoplasmic enzyme activities D-lactic dehydrogenase and succinic dehydrogenase (not shown). Therefore, these two fractions can be assumed to be cytoplasmic membranes. Together they contain more then 50% of the total membrane lipid in both wild-type and auxotrophic cells grown in 18 : 1. Depletion of the lipids in L_1 may account for the slight increase in protein/lipid ratios in unseparated membranes. This is an observation we have made previously with 8,8-$^{19}F_2$14 : 0 labeled *E. coli* auxotrophs [22].

Membrane ultrastructure by freeze-fracture electron microscopy

The membrane structures of CM2200 and CM5203 grown in 18 : 1, 16 : 1, and those grown in these two fatty acid supplements and later transferred to 8,8-$^{19}F_2$14 : 0 have been investigated by freeze-fracture electron microscopy. The cells were freeze-quenched from 37 or 20°C. In most cases, the fracture plane runs between the inner membrane, exposing either the E or the P faces of this membrane. Intramembranous protein particles are easily distinguishable on these fracture planes. Since the bacteria are rod-shaped, the E and P faces can be identified by their curvatures. Fig. 2A shows a typical replica of CM2200 grown without supplement and frozen from 20°C. Intramembranous protein particles from both P and E faces are not patched. The P face has a higher density of IMP than the E face. When the fracture plane runs along the outer membrane, a 'matt' surface with tightly packed structures representing the peptidoglycan is observed (Fig. 2B).

CM5203 cells grown in 18 : 1, 16 : 1, or those later transferred to 8,8-$^{19}F_2$14 : 0 show different

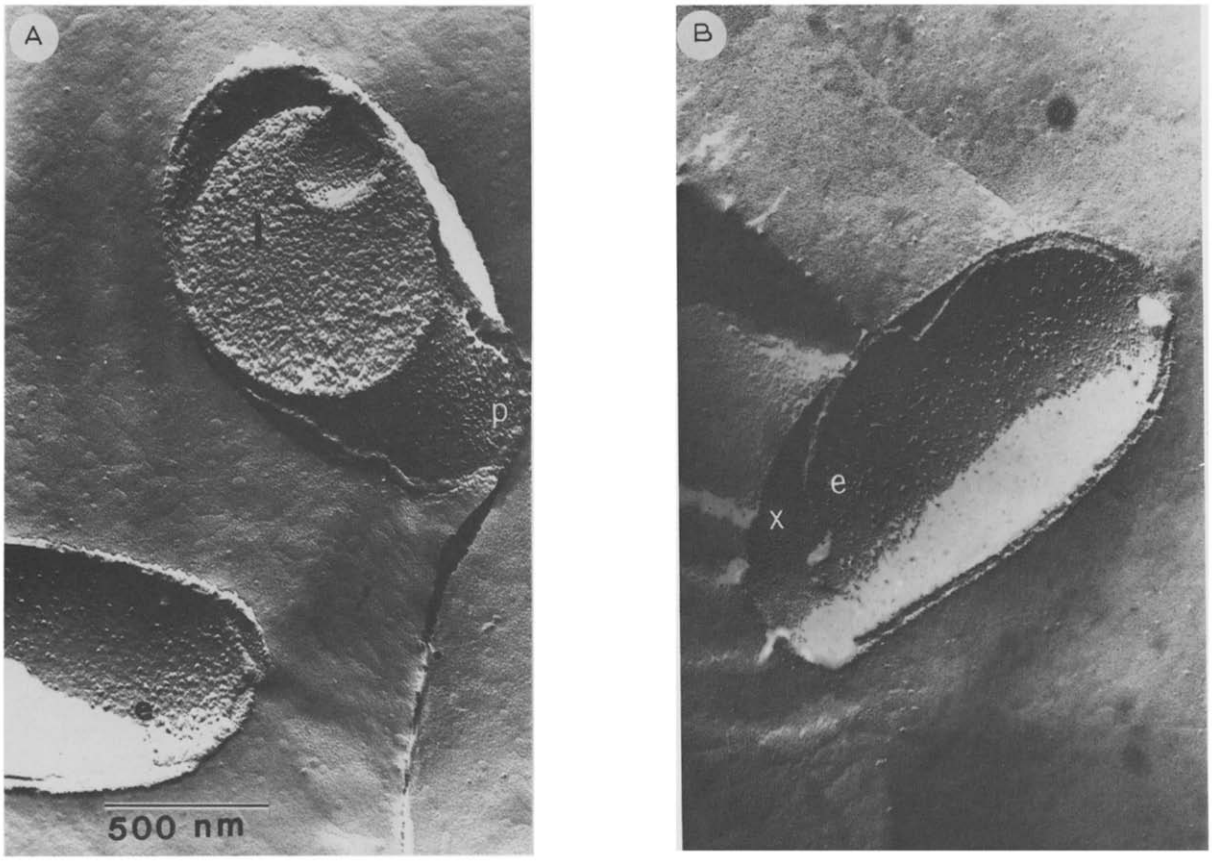

Fig. 2. Freeze-fracture electron micrographs of CM2200 grown without supplement and freeze-quenched from 20°C. (A) E and P faces are indicated and I indicates interior of the cell; and (B) Fracture face of the outer membrane is indicated by X. Bar = 500 nm.

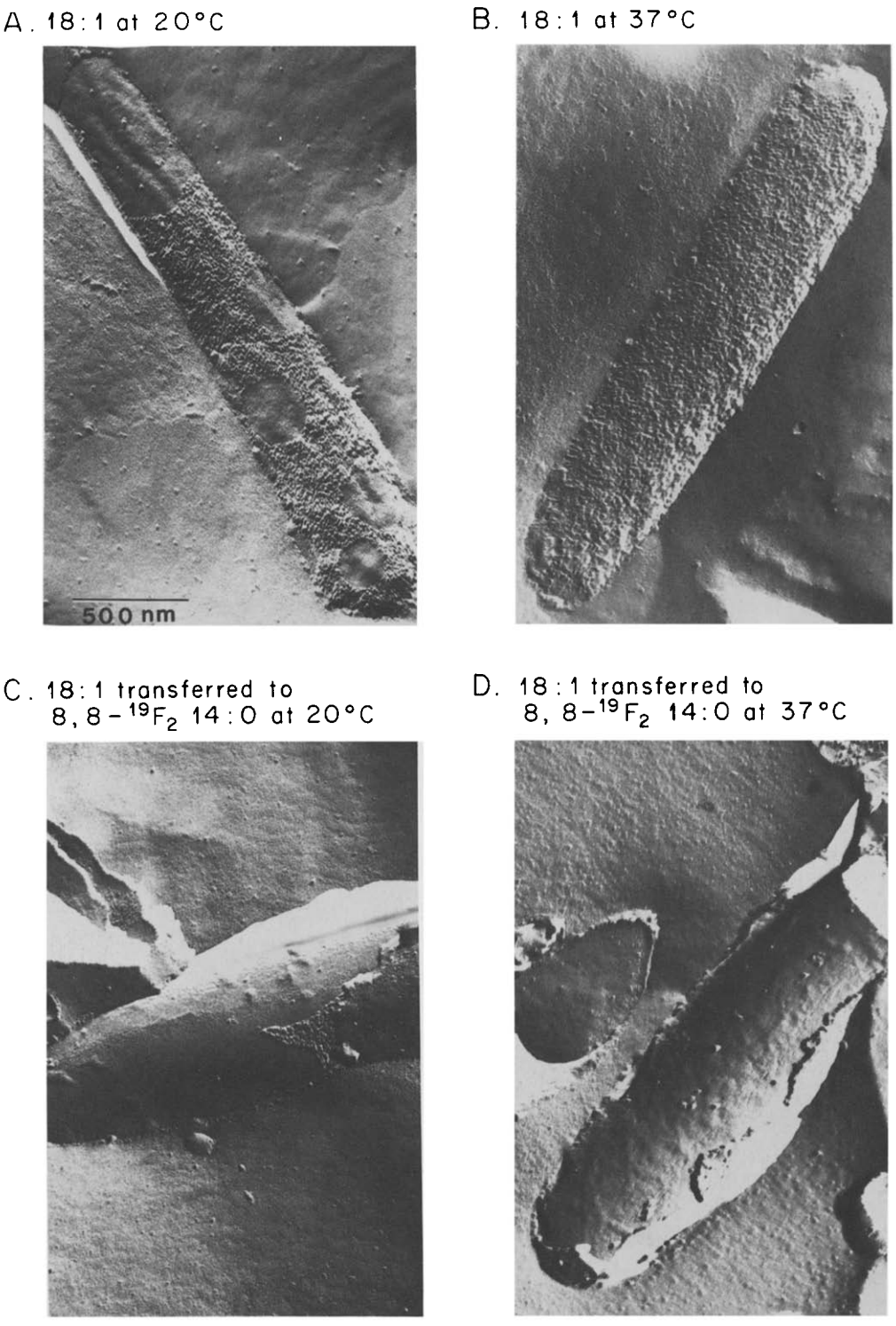

Fig. 3. Freeze-fracture electron micrographs of the P face of CM5203 as a function of fatty acids and freeze-quenching temperature (A) 18:1 at 20°C; (B) 18:1 at 37°C; (C) 18:1 transferred to 8,8-$^{19}F_2$14:0 at 20°C; and (D) same as in (C) but at 37°C.

intramembranous protein particle distributions. The intramembranous protein particle distributions also change with the freeze-quench temperature. Fig. 3 shows the intramembranous protein particle distribution on the P face of CM5203 grown in 18:1. Quenched from 20°C, many intramembranous protein particle-denuded patches are observed (Fig. 3A). These areas all but disappear if the samples are quenched from 37°C (Fig. 3B). The appearance of these intramembranous protein particle-denuded patches is similar to those observed in *E. coli* ML308-225 fatty acid auxotroph membranes under identical treatments (results not shown). The density of intramembranous protein particles on the remaining area of patched membranes is higher than the average intramembranous protein particle density on the unpatched membranes. CM5203 cells grown in 18:1 and then transferred to 8,8-$^{19}F_2$14:0 show extreme intramembranous protein particle patching at all temperatures (Fig. 3C and D). The intramembranous protein particle-denuded

TABLE IV

FREEZE-FRACTURE MORPHOLOGY OF THE INNER MEMBRANE OF CM2200 AND CM5203

Freeze-quenching temperature (°)	Strain	Supplement	Degree of intramembranous protein particle patching [a]
37	CM5203	16:1	–
		8,8-$^{19}F_2$14:0	++
	CM5203	18:1	–
		8,8-$^{19}F_2$14:0	++
20	CM5203	16:1	–
		8,8-$^{19}F_2$14:0	+++
	CM5203	18:1	+
		8,8-$^{19}F_2$14:0	+++
	CM2200	none	–

[a] The degree of intramembranous protein particle patch is visually classified as: –, no patching; +, slight; ++, moderate; +++, extreme patching.

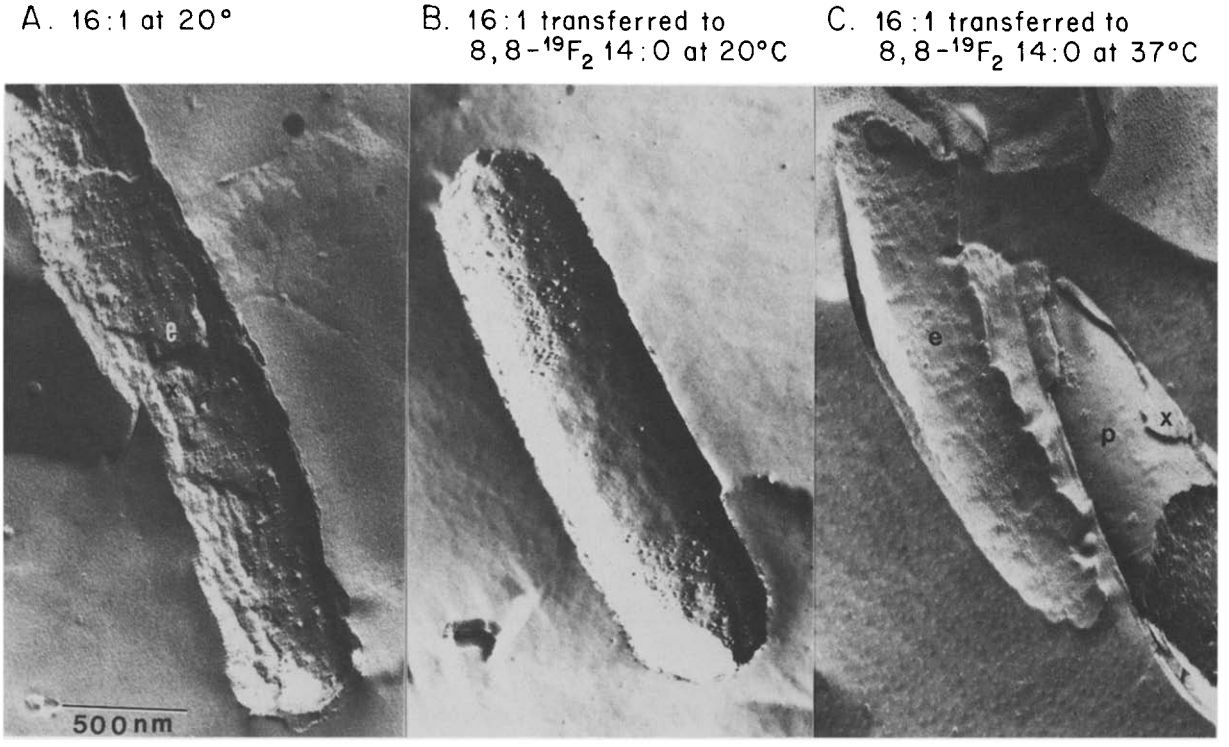

Fig. 4. Freeze-fracture electron micrographs of the E face of CM5203 as a function of fatty acids and freeze-quenching temperature: (A) 16:1 at 20°C; (B) 16:1 transferred to 8,8-$^{19}F_2$14:0 at 20°C; and (C) same as in (B) but at 37°C.

patches are so extensive that only a small fraction of the fractured face has intramembranous protein particles. The degree of intramembranous protein particle patching or aggregation is visually graded and tabulated in Table IV. Whatever intramembranous protein particle patching is observed on the P face, corresponding patching can also be observed on the E face.

CM5203 cells grown with 16 : 1 show similar patching behavior except that patching is not apparent when quenched from 20°C (Fig. 4A). However, if the bacteria are later transferred to 8,8-$^{19}F_2$14 : 0, significant patching is again observed at all temperatures (Fig. 4B and C). E face micrographs are shown in Fig. 4 since examples of P face appearance are already given in Fig. 3. The response of individual bacteria to temperature and lipid supplement changes are uniform through each sample.

β-Galactosidase and transport experiments

In *E. coli*, the active transport of β-galactosides has been the most extensively studied of microbial transport systems and has recently been successfully reconstituted into liposomes [38,39]. In *Salmonella,* transport of the β-galactoside methyl thio-β-D-galactopyranoside is a sodium-dependent α-galactoside transport system [40] and has received relatively little attention. Assay procedures for β-galactosidase can be easily and rapidly performed. Its introduction into *S. typhimurium* and the auxotrophic derivatives gave us a simple procedure for measuring cell lysis by the presence of enzyme activity in the external medium and allowed us to compare the effect of 8,8-$^{19}F_2$14 : 0 on transport by *Salmonella* to that observed in *E. coli*.

Fig. 5A and B show that β-galactosidase can be induced in an organism deprived of an unsaturated fatty acid or during incorporation of 8,8-$^{19}F_2$14 : 0, meaning that no particular fatty acid component is important for sufficient quantity of the small molecular weight compound to enter the cell and induce synthesis of the enzyme [11]. Hydrolysis of *o*-nitrophenyl galactopyranoside follows the time-course of growth at 38 and 20°C according to the supplement provided. That the membrane remains intact during this period is apparent, as there is no cell lysis, indicated by the absence of enzyme activity in culture supernatants.

Transport experiments with resting cells fully

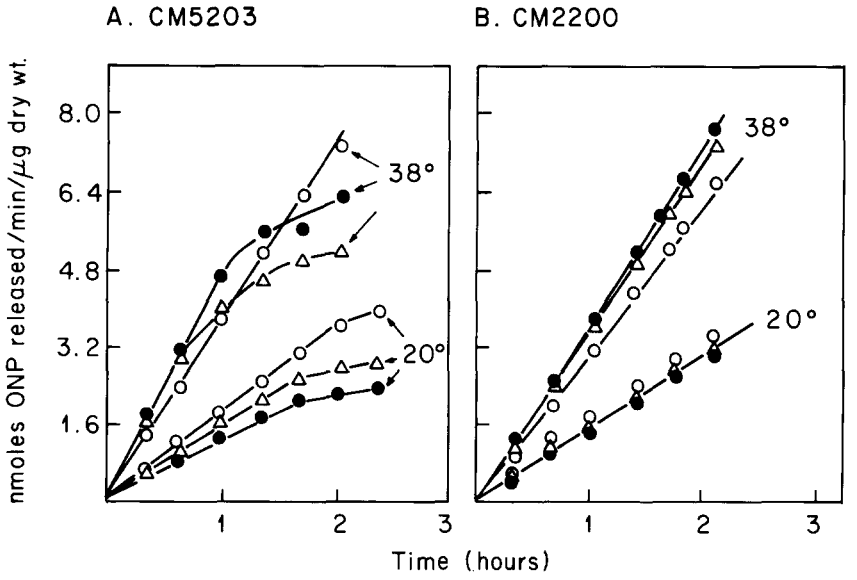

Fig. 5. Induction of β-galactosidase in *S. typhimurium* as a function of fatty acids and temperature: (A) CM5203; and (B) CM2200. Cells were grown to log phase in minimal glycerol containing 16 : 1 or 18 : 1, washed, resuspended and induced with isopropyl β-thiogalactopyranoside as described. The upper curves are those obtained at 38°C, the lower ones at 20°C. ○, cells supplemented with 16 : 1 or 18 : 1; ●, cells transferred to 8,8-$^{19}F_2$14 : 0; △, cells with no addition.

induced by growth on lactose supplemented with 18 : 1 or 16 : 1 show that at steady state, the internal concentration of methyl thio-β-D[^{14}C]galactopyranoside is lower in CM5203 than in CM2200 grown under identical conditions. The results obtained from cells grown at 38°C and assayed at 38, 30 and 20°C can be seen in Fig. 6. The concentration of 8,8-$^{19}F_2$14 : 0 in the lipids of CM5203 after 0.8 generations is approx. 16% and the unsaturated fatty acid concentration is 18–25%. These cells show a marked reduction in the ability to accumulate methyl thio-β-D-galactopyranoside. Transport by CM2200 is not influenced by the small amount (4–5%) of 8,8-$^{19}F_2$14 : 0 incorporated at this temperature, nor is it affected by the 10–11% incorporated at 20°C (not shown). The difference in accumulation at steady state between the two strains is greater as the temperature of the transport assay is reduced. Initial rates of transport, as determined by 30-s samples, show a similar decline by CM5203 with decreasing temperature. The reduction in the initial rate of uptake and of accumulation at steady state by the fatty acid auxotroph, as a percentage of the values for CM2200, is given in Table V. In Fig. 6A, it can be seen that, assayed at 38°C, the initial entry of methyl thio-β-D-galactopyranoside into CM5203 containing 8,8-$^{19}F_2$14 : 0 is followed by a rapid exit and the cells reach a steady-state equilibrium at an internal concentration 50% that of those cells grown in 18 : 1. The 'overshoot' is not apparent at 30 or 20°C. This, and the data in Table V, imply that the two processes can be separated and that the fatty acid content and temperature, which influence the physical state of

TABLE V

TRANSPORT AND ACCUMULATION OF METHYL THIO-β-D-GALACTOPYRANOSIDE BY CM5203 EXPRESSED AS A PERCENTAGE OF THE VALUES OBTAINED FOR CM2200

Temperature (°C)	Supplement	Initial rate [a]	Steady-state concentration [b]
38	18 : 1	80	80
	8,8-$^{19}F_2$14 : 0	55	40
30	18 : 1	79	74
	8,8-$^{19}F_2$14 : 0	39	26
20	18 : 1	50	35
	8,8-$^{19}F_2$14 : 0	24	10

[a] μmol methyl thio-β-D-[^{14}C]galactopyranoside transported/g dry wt. of cells per min. Determined by 30-s samples.

[b] μmol methyl thio-β-D-[^{14}C]galactopyranoside accumulated at steady state/g dry wt. of cells.

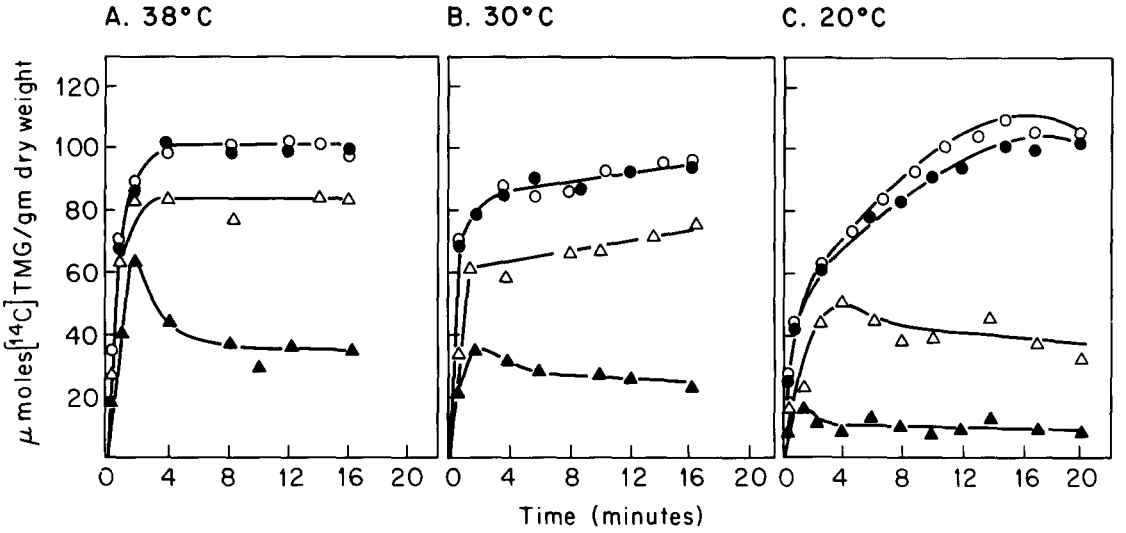

Fig. 6. Uptake of methyl thio-β-D-[^{14}C]galactopyranoside by *S. typhimurium* CM2200 and CM5203 as a function of fatty acids and temperature: (A) 38°C; (B) 30°C; and (C) 20°C. Cells were grown to log phase at 38°C in minimal lactose, histidine supplemented with 18 : 1, washed and resuspended, and growth was continued for 0.8 generations. ○, CM2200 grown in 18 : 1; ●, CM2200 grown in 8,8-$^{19}F_2$14 : 0; △, CM5203 grown in 18 : 1; ▲, CM5203 grown in 8,8-$^{19}F_2$14 : 0.

the membrane lipids, have a greater effect on exit from the cell than on the entry process. In auxotrophs both of these processes are disrupted by the depletion of unsaturates.

Fig. 7 shows the efflux of radioactive label from cells pre-loaded with methyl thio-β-D-[^{14}C]galactopyranoside following addition to non-radioactive lactose. There is a rapid loss of radioactivity from CM2200 and CM5203 grown in 18 : 1 which cannot be observed in CM5203 containing 8,8-$^{19}F_2$14 : 0. Efflux occurs under exchange conditions and is dependent on the internal substrate concentration [41]. 8,8-$^{19}F_2$14 : 0 labeled auxotrophs are unable to maintain sufficient internal concentration for exchange of the two β-galactosides at all temperatures.

Cells induced by growth in melibiose accumulate lower levels of methyl thio-β-D-galactopyranoside, but the pattern is the same (results not shown).

Transport of tetracycline, too, is an energy dependent carrier-mediated process [42–45]. Resistance is the result of decreased accumulation due to a rapid efflux of the antibiotic brought about

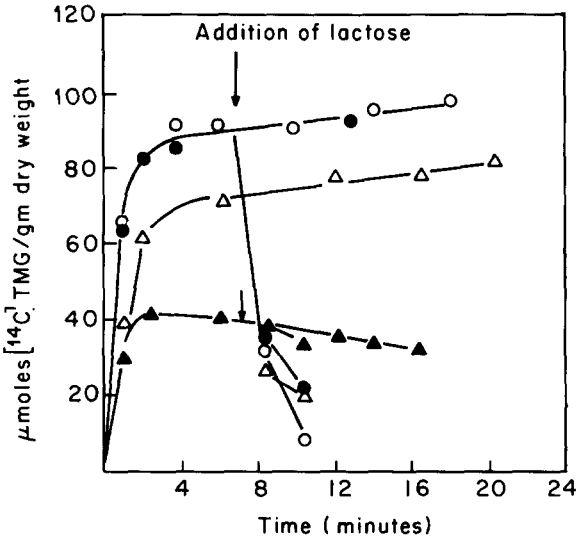

Fig. 7. Uptake and chase of methyl thio-β-D-[^{14}C]galactopyranoside by *S. typhimurium* CM2200 and CM5203 at 30°C. Cells were grown at 38°C in minimal lactose containing histidine and 18 : 1, washed, and resuspended in 18 : 1 or 8,8-$^{19}F_2$14 : 0 for 0.8 generations. The arrow at 7 min indicates the addition of lactose. ○, CM2200 in 18 : 1; ●, CM2200 in 8,8-$^{19}F_2$14 : 0; △, CM5203 in 18 : 1; ▲, CM5203 in 8,8-$^{19}F_2$14 : 0.

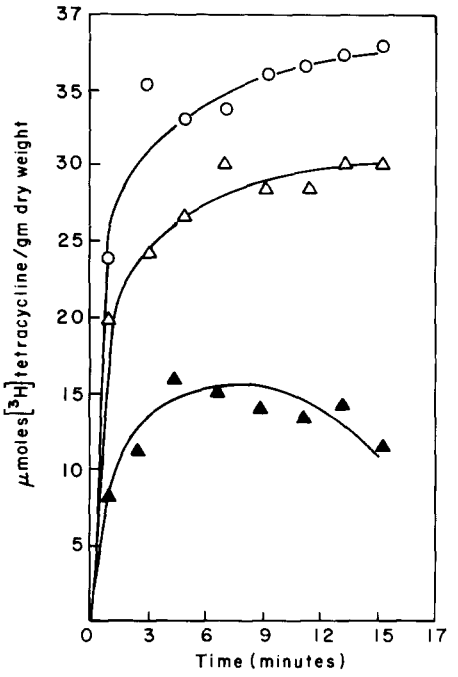

Fig. 8. Uptake of [^{3}H]tetracycline by *S. typhimurium* TA1859 and CM5003 as a function of fatty acids at 37°C. ○, TA1859 in 18 : 1; △, CM5003 in 18 : 1; ▲, CM5003 in 8,8-$^{19}F_2$14 : 0. Cells were grown in minimal melibiose to exponential phase, washed and resuspended, and growth was continued for 0.8 generations. The reaction was started by the addition of 50 μM [^{3}H]tetracycline.

by the synthesis of membrane proteins [46]. Fig. 8 shows the accumulation of tetracycline in sensitive cells of *S. typhimurium* TA1859 and CM5003. Steady-state levels in the unsaturated fatty acid auxotroph are approx. 74% those of TA1859 when the cells are grown in 16 : 1 or 18 : 1 and 30% when the cultures have been shifted to 8,8-$^{19}F_2$14 : 0.

Distinct from the carrier-mediated processes is the transport of L-histidine across the membrane of *S. typhimurium* accomplished through participation of the histidine-binding protein J with three other membrane-associated proteins P, M and Q [47,48]. We have found histidine transport is not reduced in the fatty acid auxotroph with any of the fatty acid supplements provided. Over the time-course of the transport assays, levels obtained by CM5203 are consistently higher than those of CM2200. This may be explained by the fact that the assays must be performed using intact cells which overproduce J protein and in the

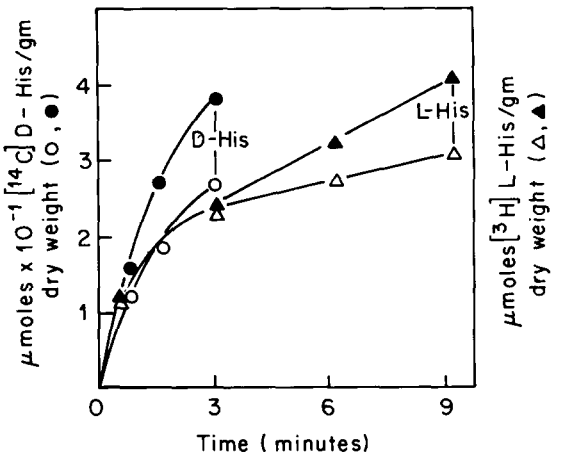

Fig. 9. Uptake of D and L-histidines by *S. typhimurium* CM2200 and CM5203 at 37°C. For D-histidine, ○, CM2200 and ●, CM5203. For L-histidine, △, CM2200 and ▲, CM5203.

case of the auxotroph, the periplasmic component of the system is excreted from the periplasmic space (see below), possibly binding the substrate. The determinations, then, would not necessarily be a measurement of the transport process. It is also possible that, indeed, some protein-lipid interactions are taking place in these cell membranes which enhance transport activity. The results can be seen in Fig. 9.

Excretion of periplasmic proteins and sensitivity to inhibitory agents

Similar to the phenotypic characteristics of the *lky* class of mutants found in *Salmonella* (24,36), fatty acid auxotrophs of *S. typhimurium* excrete or leak proteins from the periplasmic space during growth. This occurs at 20, 30 or 37°C and regardless of the fatty acid provided to satisfy the requirement. Excretion of these periplasmic proteins from colonies of the parent strain TA1859 does not occur. There are, in addition, differences in sensitivity between the mutant and wild-type strains to both tetracycline and ethidium bromide. CM5003 can tolerate more tetracycline than TA1859 and ethidium bromide has no effect on the growth of the auxotroph in quantities at least 10-fold greater than that inhibitory to TA1859.

Excretion of periplasmic proteins implies that an important functional property of the outer membrane has been disrupted by alterations of some physical or structural property and that these are not reflected by the parent strain as a response to changes in the relative fatty acid concentrations of the membrane lipids resulting from temperature or environmental adjustments. The results are summarized in Table VI.

TABLE VI

SENSITIVITY TO INHIBITORY AGENTS AND EXCRETION OF PERIPLASMIC PROTEINS

Strain	Sensitivity to inhibitory agents (μg/ml) [a]			Excretion of proteins	
	ethidium bromide	tetracycline	tetracycline [b]	RNAase	J protein
TA1859	49	0.4	0.6	no	no
CM5003	> 400	1.7	1.5	yes	yes

[a] μg/ml required for a 50% reduction in growth rate.
[b] μg/ml required for a 50% reduction in colony-forming units.

Increased resistance to tetracycline and ethidium bromide may be the result of either impairment of transport or increased efflux.

Discussion

^{19}F-NMR studies of microbial membrane structure-function relationships have been concerned mainly with the cytoplasmic membranes of fatty acid auxotrophs of *E. coli* [21,22], the plasma membrane of yeast [49] and *Acholeplasma laidlawii*, an organism with only a plasma membrane which, under certain conditions, will incorporate monofluoropalmitic acids as spectroscopic probes [50]. Our concern with the application of ^{19}F-NMR as an informative spectroscopic probe for monitoring protein-lipid interactions prompted us to isolate fatty acid auxotrophs of *S. typhimurium* and look at a few of their membrane properties influenced by lipid structure.

Among the many physiological functions of the outer membrane of Gram-negative bacteria are those of providing a diffusion barrier for some compounds such as antibiotics, pores that permit the free diffusion of certain low-molecular-weight compounds, receptor sites for phages and colicines. It participates in conjugation, cell division, septum formation and is involved with maintaining the structural integrity of the cell. In addition,

the outer membrane provides a protective environment for hydrolytic enzymes and other periplasmic proteins.

Dependence on an external supply of fatty acids or the inability to synthesize them in the proper proportions required for any particular temperature results in enough disruption of the outer membrane structure to impair some essential functions of that membrane. Auxotrophs excrete of leak the periplasmic proteins RNAase and histidine-binding protein J. An additional observation is worth mentioning here. We have isolated a variant of CM5203 able to grow without a fatty acid supplement. The organism synthesizes very small quantities of 16 : 1 and 18 : 1 but sufficient to maintain growth at a very slow rate. When grow in either supplemented or unsupplemented media, the organism excretes periplasmic proteins into the external medium. HFR derivatives of this and fatty acid auxotrophs are efficient donors in conjugational crosses; however, all the auxotrophs we have tested are very poor recipients in such crosses, illustrating another impairment of the outer membrane.

Microbial growth is under complex genetic and physiological control and influenced by environmental conditions. Septum formation and division of the membranes must be controlled both spatially and temporally for a cell to retain its characteristic shape during growth. Growth rates are reduced in auxotrophic strains, even though the fatty acid substitution is 16 : 1 which at, at temperatures tested, is synthesized in relatively constant amounts by the parent organism. Substitution of 18 : 1 ($\Delta 9 : 10$) for native *cis*-vaccenic acid ($\Delta 11 : 12$) produces the same result. 14 : 1 ($\Delta 9 : 10$) has a more marked effect on growth rates possibly because of chain length differences or a difference in the thermotropic properties of the membrane lipids enriched with this component. The quantity of *cis*-vaccenic acid is synthesized in increasing amounts by wild-type cells as the growth temperature is reduced. When available in the external medium, 16 : 1 and 18 : 1 are both incorporated by wild type cells changing their relative proportions in the lipids. Unlike auxotrophic cells, a shift in these proportions has no deleterious effect on cellular activity. Our results show that altering the fatty acid content of membrane lipids causes morphological differences between auxotrophic and wild-type cells. These are exaggerated considerably when the fatty acid requirement is removed and, to a lesser extent, in the presence of 8,8-$^{19}F_2$14 : 0. Whatever the molecular mechanism(s) involved the fatty acid components of the membrane lipids play an important role in determining morphological characteristics.

On the ultrastructural scale, the degree of patching of intramembranous protein particle in the cytoplasmic membrane increases in the order of parent < 16 : 1 < 18 : 1 < 8,8-$^{19}F_2$14 : 0. The patch formation depends on temperature as well. The thermotropic patching of intramembranous protein particles in *E. coli* is interpreted as an indication of lipid phase separation [51,6,8]. With the same reasoning, our morphological findings on *Salmonella* indicate the onset temperature for membrane lipid separation is lowered by the same order as that shown above. The temperature-dependent experiment supports this interpretation. CM5203 cells grown in 16 : 1 have a lower onset temperature ($T < 20°C$) than those grown in 18 : 1 ($20 < T < 37°C$). When either is transferred to 8,8-$^{19}F_2$14 : 0, the onset temperature is raised to more than 37°C. This change in the physical properties is reflected in the function of the bacteria as discussed below.

Transport experiments demonstrate clearly that, similar to *E. coli*, transport of β-galactosides is influenced by the lipids contained in the membranes. The use of isogenic strains shows that steady-state levels obtained by the auxotroph are consistently lower than those of the parent strain. Whether this is the result of a general metabolic disturbance or an altered physical state of the lipids requires further study. However, data obtained from electron micrographs of specimens freeze-fractured following quenching from 37 and 20°C strongly support the latter hypothesis.

At 38°C, CM5203 cells grown in 18 : 1 are above the onset for lipid phase separation, while those transferred to 8,8-$^{19}F_2$14 : 0 are still below the onset. At 20°C, both samples are below their respective onsets (Figs. 2 and 3). This is reflected in their methyl thio-β-D-[^{14}C]galactopyranoside uptakes. When assayed at 38°C, the uptake of the 18 : 1 sample is closer to that of the 8,8-$^{19}F_2$14 : 0-containing sample (Fig. 6A and C). At 20°C, the

uptakes of both samples are considerably lower than those of CM2200 whose membranes are still at a state above the onset of lipid phase separation (Fig. 2). The significant differences in the membrane ultrastructure of CM5203 before and after the cells are transferred to 8,8-$^{19}F_2$14 : 0 may also be related to their different responses to the addition of lactose to preloaded cells (Fig. 7). None of these effects can be attributed to the presence of ethanol or Brij 58 in the medium [51,52] or they could be observed in the wild-type organism as well.

We have made an attempt to assess the suitability of 8,8-$^{19}F_2$14 : 0 as a spectroscopic probe for studying membrane protein-lipid interactions. In conclusion, it appears that the substitution of this analog for 18 : 1 or 16 : 1 is not in itself a perturbing molecular probe in the membrane. Its presence in membranes of wild-type cells has no apparent or significant effect on any of the membrane properties that we have measured, although at low temperatures it is incorporated in significant amounts. In the case of unsaturated fatty acid auxotrophs, it should be used with caution. It does not replace completely nor satisfy fully the requirement for an unsaturated fatty acid. Thus, the results presented in this paper have confirmed our earlier ^{19}F-NMR studies on *E. coli* membranes that unsaturated fatty acid auxotrophs of Gram-negative bacteria can respond to the presence of a difluoromyristic acid in a manner similar to their response to a double-bond containing fatty acid [21,22], but it does not fully satisfy their unsaturated fatty acid requirements. Some membrane properties are highly dependent on the physical state of the membrane lipids for optimal activity. Depletion of unsaturates in auxotrophs causes a marked reduction or impairment of these particular activities. Others, such as the high-affinity transport system of L-histidine, are not so dependent and remain fully functional.

Acknowledgements

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