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A Density Label for Membranes

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Abstract. Bromostearic acid satisfies the essential fatty acid requirement of an unsaturated fatty acid auxotroph of *Escherichia coli* K12. Over 55% of the fatty acids derived from lipids isolated from membranes of cells grown in bromostearate-supplemented medium contained bromine, as established by gas chromatography and mass spectrometry of the methyl esters. During equilibrium centrifugation in sucrose gradients, the cytoplasmic membranes isolated from bromostearate-grown cells banded at a density approximately 0.06 g/cm^3 greater than membranes isolated from oleate- or elaidate-grown cells, affording a complete separation between membranes which contain brominated fatty acids and those which do not.

Certain events in membrane biogenesis, such as the formation of septal structures during cell division, may involve local growth of membrane. In order to study this and other questions related to local membrane synthesis on the molecular level, a procedure is needed that can distinguish newly-made membranes from old. This report describes a promising technique that employs brominated fatty acids as density labels for membranes in a fatty acid auxotroph of *Escherichia coli* K12.

Materials and Methods. Fatty acids: A mixture of 9-bromo- and 10-bromostearic acids was produced by addition of HBr to oleic acid by the method of Jungermann and Spoerri.[1] The crude product was recrystallized twice from acetone at −20°C to give a white crystalline solid mp 43–44°C, uncorrected. A small sample of the product was converted to the methyl ester by reaction with diazomethane. Gas chromatography (GLC) on a 6 ft by 1/4 inch column packed with 3% SE-30 on Gas Chrom Q (Applied Science Laboratories, State College, Pa.), with a Hewlett-Packard F and M 402 instrument (oven set at 200°C) indicated that this product was at least 95% pure. Oleic (*cis*-Δ^9-octadecenoic) acid was purchased from the Hormel Institute, Austin, Minn.; and elaidic (*trans*-Δ^9-octadecenoic) acid from Aldrich Chemical Corp., Milwaukee, Wis.

Bacterial strains and media: Strains 30$^-$ and 30E are unsaturated fatty acid auxotrophs of *E. coli* K12. Strain 30$^-$ is described elsewhere.[2–4] Strain 30E is an elaidate mutant of 30$^-$ obtained as described by Schairer and Overath.[5] Growth was in medium A,[6] with 1% Difco casamino acids as the carbon source, 5 μg/ml of thiamine-HCl, 0.25% of the nonionic detergent Triton X-100 (Rohm and Haas), and the essential fatty acid (oleate, elaidate, or bromostearate) at 0.02%.

Growth of cultures: Cell growth was at 37°C with vigorous rotary shaking, and was monitored turbidimetrically.

The cells used in these studies were grown exponentially in media supplemented with the stated fatty acid for at least 5 generations.

Preparation of lipid extracts: A 500-ml culture of strain 30E was grown to 10^9 cells/ml in bromostearate medium. Cells were collected by centrifugation at 10,000

$\times g$ for 10 min, and were washed twice with a mixture of 1% KCl and 0.25% Triton X-100 and once with water. Lipids were extracted by the method of Kanfer and Kennedy[7] and concentrated to dryness by rotary evaporation *in vacuo*.

Characterization of fatty acids: Lipid extracts were hydrolyzed by dissolving the sample in 10 ml of methanol, chilling to 0°C and adding 10 ml of cold 2 N aqueous KOH. The contents were thoroughly mixed and allowed to stand for 14 hr at 4°C. The mixture was then acidified in the cold by the addition of 6 N HCl to a pH below 2. Fatty acids were extracted with ether, and the ether extracts were washed with water, dried over Na_2SO_4, and treated with diazomethane to form methyl esters. The solvent was removed and the esters were subjected to GLC and mass spectrometry. Mass spectra were taken by Dr. James McCloskey, Baylor Medical School, Houston, Texas.

Assay of β-glucoside transport: The transport of β-glucosides was determined as described previously by measuring the rate of *p*-nitrophenyl-β-D-glucopyranoside hydrolysis by intact cells.

Preparation and isopycnic banding of membranes: Exponential phase cells were harvested by centrifugation and washed once with 10 mM Tris–HCl buffer, pH 7.6. For a 500-ml culture grown to a density of 10^9 cells/ml, the washed cell pellet was suspended in 100 ml of a solution containing 0.5 M sucrose, 10 mM Tris–HCl, pH 7.6, and 10 mM 2-mercaptoethanol. The cells were converted to spheroplasts by the method of Birdsell and Cota-Robles.[8] The spheroplasts were centrifuged at 27,000 $\times g$ for 15 min, and the pellet was suspended in 10 ml of 0.5 M sucrose. This suspension was rapidly diluted with 120 ml of a solution containing 10 mM Tris–HCl pH 7.6, 3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 40 μg/ml of DNase. This preparation was homogenized with a glass–Teflon mortar and pestle to assist in rupturing of the spheroplasts, and was incubated for 30 min at 30°C to degrade DNA. The crude membrane fraction was centrifuged for 40 min at 69,000 $\times g$ in a Spinco 50.1 rotor, washed twice by suspension in 120 ml of a solution containing 10 mM Tris–HCl, pH 7.6, 10 mM 2-mercaptoethanol, and 3 mM EDTA, and centrifuged at 69,000 $\times g$. The washed membranes were suspended in, and dialyzed at 4°C overnight against, 1.5 liters of this same buffer.[9]

The dialyzed membranes were centrifuged at 80,000 $\times g$ for 60 min, and the pellet was suspended in 6 ml of 10 mM Tris–HCl, pH 7.6–10 mM 2-mercaptoethanol. 1 ml of membrane suspension was layered on each of six discontinuous sucrose gradients consisting of 1 ml of 65% sucrose and 3.0 ml of 35% sucrose. All sucrose solutions contained 10 mM Tris–HCl, pH 7.6 and 10 mM 2-mercaptoethanol. The tubes were centrifuged at 165,000 $\times g$ for 2 hr in a Spinco SW50.1 swinging bucket rotor, and the purified membrane fraction was recovered at the interface between the 35 and 65% sucrose layers. The purified membrane fraction was further separated into three fractions on discontinuous sucrose gradients consisting of 1.4 ml of 65% sucrose, 1.3 ml of 60% sucrose, and 1.3 ml of 55% sucrose. The purified membrane fraction was suspended in 6 ml of a Tris–mercaptoethanol solution, and 1 ml of this suspension was layered on each gradient. The tubes were centrifuged at 165,000 $\times g$ for 4 hr in the SW50.1 rotor. Three bands were visible, one at the top of the 55% sucrose layer, one at the 55–60% interface and one at the 60–65% interface. These are designated as bands I, II, and III respectively.

Results. Growth properties of unsaturated fatty acid auxotrophs: Unsaturated fatty acid auxotrophs of *E. coli* K12 grow in a medium supplemented with a *trans* unsaturated fatty acid only after a mutational event. Strain 30$^-$, an unsaturated fatty acid auxotroph, grows on neither bromostearate nor elaidate media. Mutation to growth in a medium supplemented with a *trans* unsaturated fatty acid also permits growth in bromostearate medium. Thus, where strain 30$^-$ grows only in oleate medium with a generation time of 85 min, strain 30E grows in oleate, bromostearate, and elaidate media with generation times of 85, 120, and 140 min, respectively.

Transport temperature profile of bromostearate grown cells: The effect of

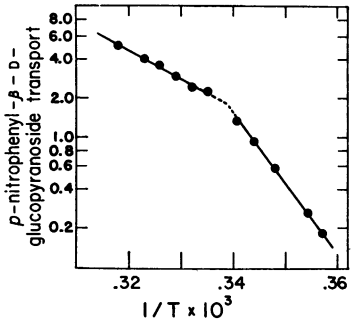

FIG. 1. The effect of temperature on transport in bromostearate-grown cells. The transition in slope (transition temperature) corresponds to 22°C.

temperature on the transport of β-glucosides is described in Fig. 1. As shown previously with cells grown on other fatty acid-supplemented media, the Arrhenius plot for transport is biphasic. The curves intersect at a transition temperature of 22°C for bromostearate-grown cells, intermediate between the transition temperatures observed for cells grown in elaidate (30°C) and oleate (13°C).[3,22]

Analysis of membrane fatty acids: The gas–liquid chromatogram of the fatty acid methyl esters of strain 30E grown in bromostearate medium is shown in Fig. 2. The major components of the fatty acid mixture were identified by mass spectrometry with an LKB 9000 spectrometer with a gas chromatographic inlet. The spectra of compounds 2 and 3 were virtually identical to reference mass spectra of methyl myristate and methyl palmitate, respectively. The spectra of components 4, 5, and 6 are presented in Fig. 3, along with the spectrum of the mixture of methyl bromostearates. These spectra may show a slight bias, since they were taken on gas chromatographic effluents. The reference compound spectrum (2*A*), which is identical to that of component 6 (2*B*) in Fig. 2, shows a pair of parent ion peaks at m/e 376 and 378. These correspond to molecules containing the two isotopes of bromine (79 and 81), and are of nearly equal intensity. The exact mass of the parent ion corresponding to the

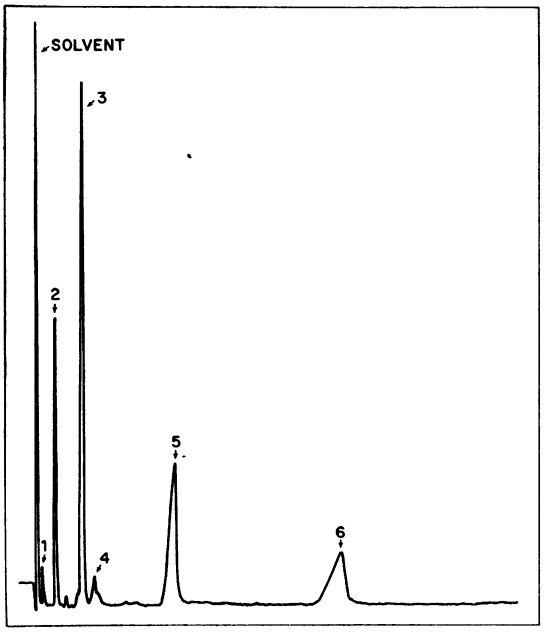

FIG. 2. Gas–liquid chromatography of fatty acid methyl esters from *E. coli* 30E cells grown in bromostearate medium, 3% SE-30 liquid phase, column temperature 200°C.

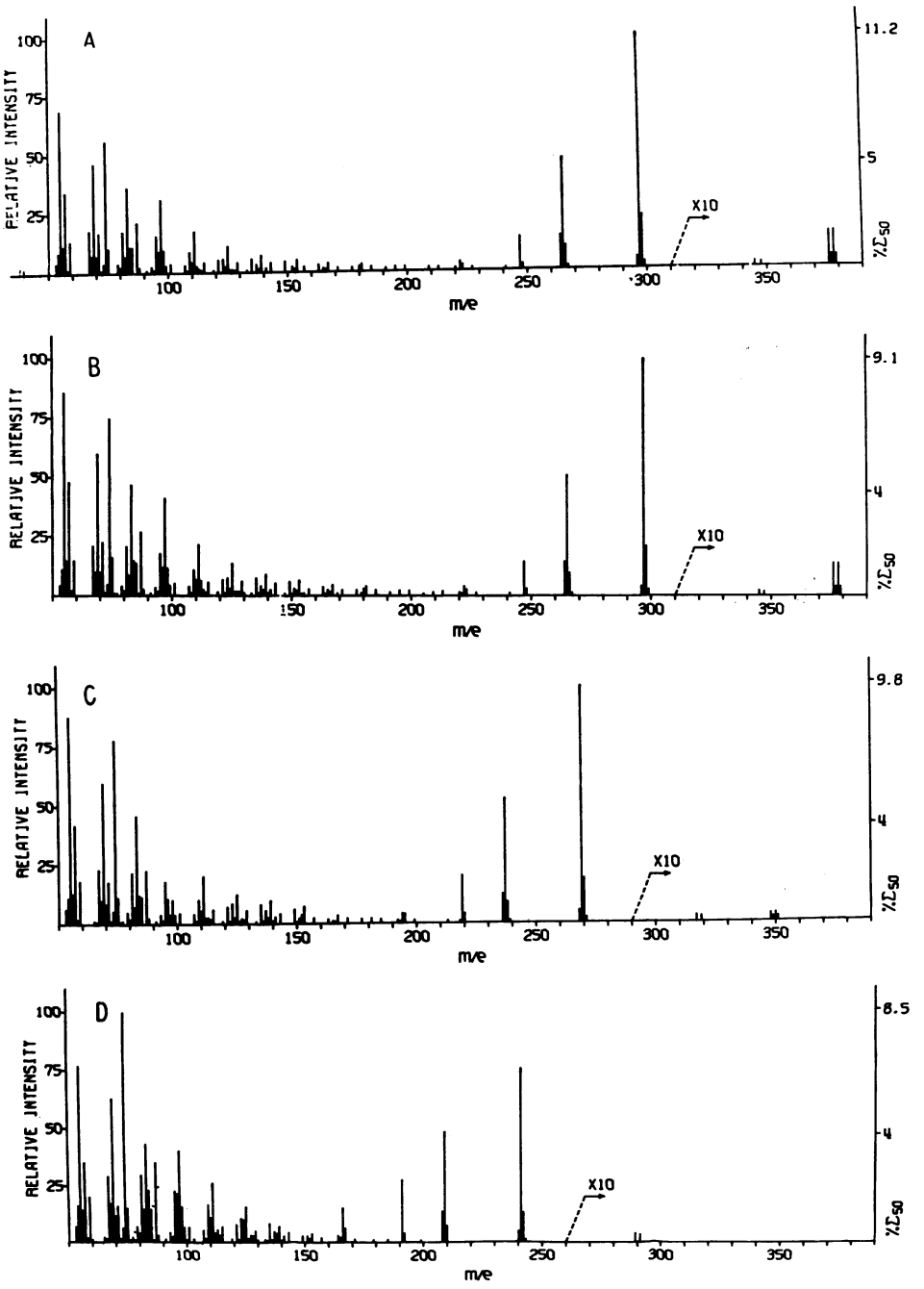

FIG. 3. Mass spectra of methyl bromostearate and methyl esters of fatty acids isolated from *E. coli* grown with 9- (and 10)-bromostearic acid. (*A*) Methyl 9- (and 10)-bromostearate; (*B*) Component 6, Fig. 2; (*C*) Component 5, Fig. 2 and (*D*) Component 4, Fig. 2.

lower bromine isotope, as measured with a CEC 21-110B high resolution mass spectrometer, was 376.1964 (calculated for $C_{19}H_{37}O_2$ ^{79}Br, 376.1976). Peaks corresponding to the loss of methoxyl from the parent ions (M minus 31) were observed at m/e 345 and 347. The base peak, m/e 297, corresponds to loss of bromine from the parent ion. Ions at m/e 265 (and 247) probably represent loss of methanol (and methanol plus water) from the base peak. The lower region of the spectrum contains hydrocarbon ions typical of fatty acid esters.

The spectra of component 5 (2*C*) and 4 (2*D*) are obviously those of lower homologs of the bromostearates. Parent ions were present in both spectra, although in 2*D* they were too weak to plot, even on the amplified scale. Ions corresponding to M minus 31, M minus bromine, M minus (bromine and methanol), and M minus (bromine, methanol, and water) are present in each case. Component 4 was contaminated with methyl palmitate, since it follows that compound in elution from the chromatograph. The parent ion of methyl palmitate, m/e 270, has not been plotted, although some other palmitate ions are present in the spectrum. Component 5 is therefore methyl bromopalmitate and Component 4 is methyl bromomyristate. These acids undoubtedly arise by β-oxidation of the administered bromostearate. The composition of the fatty acid mixture obtained from the cells is summarized in Table 1.

TABLE 1. *Fatty acid composition of E. coli mutant grown on bromostearic acid.*

Component acid	%*
Myristic (14:0)	7.4
Palmitic (16:0)	34.6
Bromomyristic (14:Br)	2.3
Bromopalmitic (16:Br)	29.2
Bromostearic (18:Br)	24.2
Unidentified	2.2

* Composition was estimated from Fig. 1 by the method of Carroll.[10]

Isolation, composition, and properties of the cytoplasmic membrane: Miura and Mizushima have described a method for separating the cytoplasmic membrane of *E. coli* from other membrane structures of this organism.[9] We have modified their method so that the cytoplasmic membranes from cells grown in oleate and bromostearate media can be isolated by identical fractionation procedures. Tables 2 and 3 describe the properties of the membrane fractions isolated by our procedure from oleate medium grown cells. Band I corresponds to

TABLE 2. *Chemical analysis and localization of cytochrome b and succinate dehydrogenase in fractionated membranes.*

Samples	Recovery of protein (%)	Recovery of carbohydrate (%)	Ratio protein/carbohydrate	Cytochrome *b*	Succinate dehydrogenase
Purified membranes	100	100	1.0	0.042	2.17
Band I	25	21	1.2	0.073	3.83
Band II	32	48	0.67	0.029	1.79
Band III	10	34	0.29	0.012	0.70

Cells were grown in oleate medium; membrane fractions were resolved as described in *Materials and Methods*. The protein and carbohydrate contents of membranes were determined according to Lowry *et al.*[11] and Dubois *et al.*[12] respectively. Cytochrome *b* was determined from difference spectra (dithionite reduced minus untreated)[13] and is expressed as the absorbance at 428 nm/mg of protein. Succinate dehydrogenase activity was determined spectrophotometrically[14] and activity is expressed as millimoles of $K_3Fe(CN)_6$ reduced per hr per mg of protein at 25°C.

TABLE 3. *Distribution of the phosphotransferases of the heat-stable protein system and M protein in fractionated membranes.*

Samples	Methyl-α-glucoside phospho-transferase	Aryl-β-glucoside phospho-transferase	Succinate dehydro-genase	M protein
Purified membranes	1.96	2.07	2.17	n.d.
Band I	4.03	3.84	3.83(3.81)	(117)
Band II	1.64	1.54	1.79(0.60)	(20)
Band III	0.59	0.06	0.70(0.55)	(10)

Cell growth, membrane fractionation, and succinate dehydrogenase assays are as in Table 2. The aryl-β-glucoside phosphotransferase and M protein were induced during three generations of growth with 0.1 mM phenyl-1-thio-β-D-glucopyranoside and 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, respectively. Phosphotransferase units are nanomoles of glycoside phosphorylated per min at 28°C per mg of protein.[16] M protein was assayed by a modification[16] of the procedure of Rumley, Armstrong, and Kennedy[17]; units are picomoles of radioactive galactoside bound to M protein per mg of protein (at half-saturating galactoside concentration). The data in parentheses are from a replicate experiment; n.d., not determined.

band A[9] and has the properties expected of the cytoplasmic membrane as shown by the presence of cytochrome *b*, succinate dehydrogenase, and proteins which play roles in glycoside transport, the β-galactoside M protein and the phosphotransferases of the system of Kundig, Ghosh, and Roseman.[18] The same qualitative results have been obtained with elaidate and with bromostearate membranes, i.e., the succinate dehydrogenase activity and the transport proteins are associated with band I.

Isopycnic banding of cytoplasmic membranes: The cytoplasmic membrane fractions (band I) from oleate- and bromostearate-grown cells were banded in linear sucrose density gradients (Fig. 4). Oleate membranes banded (4 independent preparations) at a density of 1.19 ± 0.005 g cm^{-3}. Bromostearate membranes banded (5 experiments) at a density of 1.25 ± 0.005 g cm^{-3}. Increasing the centrifugation time from 4 to 10 hr had no effect on the banding patterns. In experiments where the position of the membranes was revealed by protein determination or by the radioactivity derived from a labeled amino acid, the same result was obtained as with turbidity measurement. Since the auxotroph grows more slowly in bromostearate than in oleate medium, it is important to determine if the difference in growth rate could account for the different densities. This is an unlikely artifact since elaidate membranes band at the same

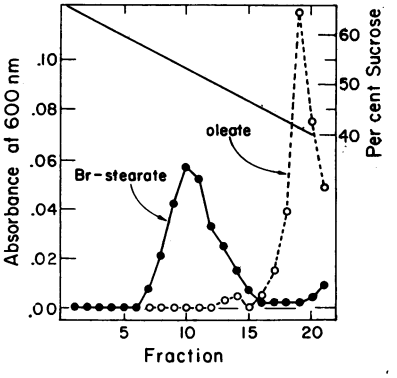

FIG. 4. Isopycnic banding of membranes in linear sucrose density gradients. The cytoplasmic membrane fractions (band I) from cells grown on oleate or bromostearate media were layered, in a volume of 0.2 ml, on top of 4.8 ml of 65–40% linear sucrose density gradient containing 10 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, and 3 mM EDTA. Centrifugation was 4 hr at 165,000 × *g* in the Spinco SW 50.1 rotor. Membranes were detected turbidimetrically by their absorbance at 600 nm, and densities were determined by weighing 0.1 ml of each fraction.

density as oleate membranes, though growth in elaidate medium is slower than growth in bromostearate medium.

Discussion. To date, the only studies which permit a distinction between newly made and pre-existing membrane are radioautographic. Morrison and Morowitz have reported that labeled palmitate incorporated into the membranes of *Bacillus megaterium* during a pulse label is largely end-localized.[19] In contrast, Mindich, quoted by Morrison and Morowitz,[19] finds that a precursor, which is incorporated solely into the lipid of *Bacillus subtilis* membranes during pulse labeling, is randomly distributed. In our opinion, Mindich's results indicate random membrane growth only to the limit of resolution of radioautography.

A brominated fatty acid was chosen as a potential density label, because we felt that the bromine atom could cause sufficient distortion in the fatty acid chain to prevent close packing and thus behave much like naturally-occurring olefinic linkages or cyclopropane rings. We also attempted to grow cells on 9,10-dibromostearic acid, but no growth occurred at 37°C. The reason for failure in this case is not yet understood. The density labeling technique described here is currently being exploited to determine if the cytoplasmic membrane of *E. coli* grows preferentially at one or a few foci. By using combined radioisotope (^{3}H and ^{14}C) and density shifts followed by controlled membrane fragmentation, it may be possible to determine not only if the membrane grows locally, but also the extent to which it grows at given foci; this last experiment is beyond the limits of resolution of radioautography.

We have shown that there exists in *E. coli* a coordinated incorporation of newly synthesized lipid and transport proteins into membrane.[16,20–22] Use of density labeling and controlled membrane fragmentation techniques may permit a characterization of membrane fragments that contain transport proteins pulse-induced after a density shift. Density labeling of membranes can also be used in conjunction with cell synchrony and isotopic labeling of periodically produced components associated with membranes such as DNA at the origin (terminus) of replication.[23,24]

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Abbreviations: GLC, gas–liquid chromatography.

[1] Jungermann, E., and P. E. Spoerri, *J. Amer. Oil Chem. Soc.*, **35**, 394 (1958).
[2] Cronan, J. E., Jr., C. H. Birge, and P. R. Vagelos, *J. Bacteriol.*, **100**, 601 (1969).
[3] Wilson, G., S. P. Rose, and C. F. Fox, *Biochem. Biophys. Res. Commun.*, **38**, 623 (1970).
[4] Epstein, W., and C. F. Fox, *J. Bacteriol.*, **103**, 273 (1970).
[5] Schairer, H. U., and P. Overath, *J. Mol. Biol.*, **44**, 209 (1969).
[6] Davis, B. D., and E. S. Mingioli, *J. Bacteriol.*, **60**, 17 (1950).
[7] Kanfer, J., and E. P. Kennedy, *J. Biol. Chem.*, **238**, 2919 (1963).
[8] Birdsell, D. C., and E. H. Cota-Robles, *J. Bacteriol.*, **93**, 427 (1967).
[9] Miura, T., and S. Mizushima, *Biochim. Biophys. Acta*, **150**, 159 (1968).
[10] Carroll, K. K., *Nature*, **191**, 377 (1961).
[11] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[12] Dubois, M., K. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Nature*, **168**, 167 (1951).
[13] Chance, B., *J. Biol. Chem.*, **202**, 383 (1953).

[14] Slater, E. C., and W. D. Bonner, *Biochem. J.*, **52**, 185 (1952).
[15] Fox, C. F., and G. Wilson, *Proc. Nat. Acad. Sci. USA*, **59**, 988 (1968).
[16] Hsu, C. C., and C. F. Fox, *J. Bacteriol.*, **103**, 410 (1970).
[17] Rumley, M. K., J. B. Armstrong, and E. P. Kennedy, *Abstracts, Lactose Operon Meeting* (New York: Cold Spring Harbor Biol. Lab., 1969), p. 26.
[18] Kundig, W., S. Ghosh, and S. Roseman, *Proc. Nat. Acad. Sci. USA*, **52**, 1067 (1964).
[19] Morrison, D. C., and H. J. Morowitz, *J. Mol. Biol.*, **49**, 441 (1970).
[20] Fox, C. F., *Proc. Nat. Acad. Sci. USA*, **63**, 850 (1969).
[21] Wilson, G., C. C. Hsu, S. P. Rose, and C. F. Fox, *Fed. Proc.*, **29**, 342 (1970).
[22] Wilson, G., and C. F. Fox, submitted to *J. Mol. Biol.*
[23] Sueoka, N., and W. G. Quinn, *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 695 (1968).
[24] Fielding, P., and C. F. Fox, *Biochem. Biophys. Res. Commun.*, in press.