unpublished results). Furthermore, it would appear that the mandibular canal of *T. gilli* affords a favorable raw material for future studies on the elusive role of isovaleric acid in lipid metabolism.

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Arrangement of Fatty Acyl Groups in Phosphatidylethanolamine from a Fatty Acid Auxotroph of *Escherichia coli**

David F. Silbert

ABSTRACT: The positional distribution of fatty acyl groups in phosphatidylethanolamine from an unsaturated fatty acid auxotroph of *Escherichia coli* has been investigated following growth of the mutant on unsaturated fatty acids of widely differing structure. *cis*-Octadecenoic acids with an ethylenic bond in the center of the hydrocarbon chain were incorporated into the 1 and 2 position of the phosphatide to an extent approaching that found in the same phospholipid from the prototrophic strain. Cis unsaturated fatty acids of shorter chain lengths or with a double bond close to the carboxyl terminus were assimilated almost exclusively into the 2 position with the result that the normal proportion of molecules containing two unsaturated residues was reduced markedly. When trans unsaturated fatty acids were provided as supplement for the auxotroph, they were found as major components of both the 1 and 2 positions. *trans*-Monoenoic and saturated fatty acids share similar physical properties. The behavior of most of these molecules containing trans unsaturated fatty acyl residues might be expected to correspond to that of a molecular species possessing only long-chain saturated fatty acids. Hence, virtually all of the heterogeneity conferred on the phospholipid by virtue of the combined presence of cis unsaturated and saturated fatty acids is lost. Starvation of the auxotroph for all types of unsaturated fatty acids led to increased synthesis and incorporation of *n*-tetradecanoic and *n*-hexadecanoic acids into the phospholipids, and to a reduction of preexisting molecules containing unsaturated residues by dilution and possibly turnover. These results are discussed in relation to their bearing on membrane function and on the specificity of fatty acyl transferases involved in phospholipid biosynthesis.

The isolation and characterization of mutants in fatty acid metabolism from *Escherichia coli* strain K-12 offer a biological system that is potentially very useful for examining controlled alterations in the physiological properties of membrane lipids in relation to their effects on membrane function (Silbert and Vagelos, 1967; Silbert *et al.*, 1968;

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Schairer and Overath, 1969; Fox, 1969; Esfahani *et al.*, 1969). Similar efforts have been extended to other systems such as *Mycoplasma laidlawii* (Rodwell, 1968; Henrikson and Panos, 1969; McElhaney and Tourtellotte, 1969; Steim *et al.*, 1969), and *Saccharomyces cervesiae* (Keith *et al.*, 1969).

This paper is concerned with characterizing the content and arrangement of fatty acyl groups in phosphatidylethanolamine from an *E. coli* unsaturated fatty acid auxotroph resulting from growth of the organism (1) with a variety of unsaturated fatty acid analogs as supplements and (2) without any suitable exogenous fatty acid for approximately one generation time. Unsaturated fatty acids in phospholipids are normally present in two types of molecules: those containing one unsaturated fatty acid, usually on the 2 position; those containing unsaturated fatty acids on both the 1 and 2 positions. The results reported in this paper indicate that it is possible to virtually eliminate (1) the synthesis of molecules containing two cis unsaturated fatty acids by supporting growth on certain *cis*-monoenoic acids, or (2) the formation of molecules containing any cis unsaturated residues by supplementing with trans analogs or by short-term exogenous fatty acid starvation.

Some reports of the physiological effect of analog substitution and short-term starvation employing *E. coli* unsaturated fatty acid auxotrophs have already appeared (Henning *et al.*, 1969; Fox, 1969; Schairer and Overath, 1969). One of these publications employed the auxotroph isolated and characterized in this laboratory (Fox, 1969). The present communication should pave the way for correlating specific lipid changes during membrane formation with the appearance of defective membrane function.

Materials and Methods

Organisms and Growth Conditions. E. coli K-12 strain Hfr 139 and an unsaturated fatty acid auxotroph derived from it (and now designated L010) were used in this study. They have been described previously as have the general conditions of growth and fatty acid supplementation (Silbert *et al.*, 1968). All mutant cultures used in these studies contained less than 0.5% revertants to fatty acid prototrophy. *Bacillus cereus* was kindly supplied by J. A. F. Opdenkamp (Utrecht).

For [^{14}C]acetate labeling of cellular lipids associated with fatty acid starvation, L010 was pregrown at 37° in 500 ml of minimal medium supplemented with *cis*-Δ^9-octadecenoate. The culture was harvested in log phase, washed with medium E containing 0.025% Tween 40 (Atlas Chemical Co.), and resuspended in fresh medium to give two separate 250-ml cultures only one of which contained *cis*-Δ^9-octadecenoate. The supplemented and deprived cultures were incubated at 37° for 30 min to allow restoration of exponential growth and the utilization of residual traces of *cis*-Δ^9-octadecenoate by the unsupplemented cells. Then 150 μCi of [^{14}C]acetate was added to both cultures. (*E. coli* converts a sizable fraction of many sources into acetate (Roberts *et al.*, 1963). The concentration of acetate in the culture medium under the experimental conditions used in this study was estimated as $1\text{–}2 \times 10^{-3}$ M by examining the extent of labeling as a function of increasing concentration of acetate of constant specific activity. On the basis of this determination the effective specific activity of acetate in the medium was approximately 0.3–0.6 μCi/μmole (5–10 mμCi/μg).) The cells were allowed to complete one doubling before they were harvested and washed. Previous studies in this laboratory and in others (Kass, 1968; Henning *et al.*, 1969; Fox, 1969) indicated that unsaturated fatty acid auxotrophs continued in normal exponential growth under conditions of fatty acid deprivation for a little more than one doubling before growth ceased.

Lipid Extraction and Phospholipid Fractionation. The lipids were extracted from the whole cells by the method of Bligh and Dyer (1959). The phospholipids were fractionated by thin-layer chromatography on activated 0.25- or 0.50-mm thick silica gel H 0.4 M boric acid plates (prepared in standard fashion from silica gel H Stahl (Merck, Darmstadt) and 0.4 M boric acid solution in place of water) using chloroform–methanol–water (85:35:4, v/v) as the developing solvent. Generally the best separation was achieved by starting the chromatography in lined *unequilibrated* tanks. The phospholipids were identified by reference to known compounds (bacterial phosphatidylethanolamine, Pierce Chemical Co., Rockford, Ill.; phosphatidylglycerol and cardiolipin, Supelco, Inc., Bellefonte, Pa.) as well as by their phosphorus content, their known relative abundance in *E. coli* in different growth phases, and the reaction of phosphatidylethanolamine with ninhydrin. The R_F values for phospholipids when present individually or in mixtures over many runs ranged as follows: phosphatidylglycerol, 0.30–0.32; phosphatidylethanolamine, 0.44–0.53; and cardiolipin, 0.70–0.78. The glycerophosphatides were extracted from the silica gel by the method of Bligh and Dyer (1959) with quantitative yields as judged by the recovery of radioactively labeled lipids.

Determination of Positional Distribution of Fatty Acyl Groups. Phosphatidylethanolamine was treated with *Crotalus adamanteus* venom (Sigma Chemical Co., St. Louis) as a source of phospholipase A, and the lysophosphatidylethanolamine and fatty acid products were separated by thin-layer chromatography according to the methods of Van Golde and Van Deenen (1967) except that Tris (0.01 M, pH 7.3) replaced borate (0.05 M, pH 7.0) as buffer in the hydrolysis reaction. The hydrolysis was continued until no further phosphatidylethanolamine remained in order to avoid complicating the results by the differential rate of release from the 2 position of different fatty acids chains. The free fatty acid and lysophosphatidylethanolamine fractions as well as phosphatidylethanolamine not exposed to the enzyme were converted into methyl esters and analyzed in a Varian Aerograph Model 2100 gas–liquid chromatograph equipped with flame ionization detectors under conditions described previously (Silbert *et al.*, 1968). The mass response was quantitated by an Infotronics Digital readout system, Model CRS-100. Appropriate care was exercised to assure that no significant contaminating lipids were derived from silica gel plates or solvents used in extraction, methylation, and chromatography.

Isolation of Diglyceride Subfractions from Phosphatidylethanolamine. Phosphatidylethanolamine (alternately, phosphatidylglycerol or cardiolipin) was converted into 1,2-diglycerides according to the following modification of the method of Haverkate and Van Deenen (1965); 75 μmoles of sodium borate, 2 μmoles of calcium chloride, and 0.5 mg of crude phospholipase C protein (prepared as described

below) in a total volume of 1 ml, final pH 7.0; 1 mg of phosphatidylethanolamine in 1 ml of diethyl ether containing sodium diethyldithiocarbamate as preservative. The biphasic system was stirred vigorously at room temperature for 30–60 min using a Vortex mixer. The reaction was continued until completion as determined by examining aliquots of the ether layer on silica gel G microslides developed with petroleum ether (bp 30–60°)–ethyl ether–formic acid (60:40:1.5, v/v). The diglyceride product was extracted with several volumes of ethyl ether. The absence of 1,3-diglyceride was confirmed by chromatography of an aliquot of the product on silica gel treated with 0.4 M boric acid according to the procedure of Thomas *et al.* (1965), using 1,2- and 1,3-dipalmitin (Applied Science, College Park, Pa.) as reference standards. The 1,2-diglyceride was fractionated as described by Van Golde and Van Deenen (1967) with silica gel G–silver nitrate plates activated at 120° for at least 4 hr immediately before use. Fractions were visualized with 0.2% dichlorofluorosein in 95% alcohol and scraped off the plate. Radioactivity was measured by counting the silica gel fractions in Bray's solution after the addition of two drops of saturated sodium chloride (to precipitate out silver chloride) using a Packard Tri-Carb liquid scintillation spectrometer, Model 3375. The counts recorded represented a counting efficiency of greater than 80% as determined by an external standard. They were converted into disintegrations per minute by manual or automatic computation. The fatty acid composition and the relative amounts of the diglyceride subfractions were established by reference to a known mass of pentadecanoic acid added as internal standard to the silica gel fractions, which were then extracted by the method of Bligh and Dyer (1959), and the lipid converted into fatty acid methyl esters and analyzed by gas–liquid chromatography. The masses of the various fatty acid esters, relative to the internal standard, were expressed as mole per cent. Formation of cyclopropane derivatives from the unsaturated fatty acyl residues in the phospholipid (which might have complicated the fractionation and analysis of the diglyceride subfractions) did not occur significantly during the period of fatty acid starvation.

Radioactivity of the individual fatty acid esters derived from the diglyceride subfractions was determined by monitoring the effluent from the Varian Aerograph gas–liquid chromatograph with a Nuclear-Chicago, Model 4998, gas radiochromatograph operating with a counting efficiency of greater than 90%. Specific radioactivities were obtained after quantitating the response of both the gas–liquid and the gas radiochromatographs with appropriate standards.

Preparation of Phospholipase C. A crude preparation of phospholipase C was obtained from the growth medium of a culture of *B. cereus* essentially as described by Haverkate and Van Deenen (1965). The trichloroacetic acid precipitable protein material in the crude preparation of phospholipase C was 8 mg/ml. The preparation was extracted with an equal volume of ethyl ether four times to remove any lipid.

Analytical Procedures. Protein was determined by the modified microbiuret method of Mokrasch and McGilvery (1956) and by the procedure of Lowry *et al.* (1951). Lipid phorphorus was measured as described by Ames and Dubin (1960). Dry weights of cells were measured after lyophilizing a washed cell pellet of approximately 5 g wet wt. Lipid weight was determined from the Bligh and Dyer extractable fractions.

Results

Glycerophosphatide Composition of Unsaturated Fatty Acid Auxotroph. GENERAL FINDINGS. The lipids of *E. coli* are more than 90% glycerophosphatides and appear to be derivatives of a common precursor, phosphatidic acid (Chang and Kennedy, 1967). The major classes of phosphatides present in log-phase cultures of the fatty acid auxotroph are found to be essentially the same as that of wild-type cells; namely, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin with phosphatidylethanolamine predominating throughout all growth phases and under all growth conditions. In the various mutant cultures no new phosphatide class has been identified.

During short-term unsaturated fatty acid deprivation (for approximately one generation time), the lipid–phosphorus (or lipid) to dry weight ratios remain essentially the same as in the unstarved state.

The studies to follow in this communication have focused on changes in the acyl residues of the predominant glycerophosphatide, phosphatidylethanolamine, in response to the various manipulations such as growth on analogs or short-term starvation.

Positional Distribution of Acyl Residues in Phosphatidylethanolamine from Cultures Supplemented with Unsaturated Fatty Acids of Normal Geometry. Table I compares the fatty acid composition by position of phosphatidylethanolamine derived from wild-type cells and from an unsaturated fatty acid auxotroph when *cis*-monoenoic fatty acids of normal geometry are present as supplements in the medium. Two different cis-18:1[1] fatty acids give essentially identical distribution of fatty acid chains in the phospholipid obtained from wild-type cells (culture 1 and 2, Table I). This is in keeping with the general observation that the composition of the wild-type *E. coli* lipid is uninfluenced by the presence of supplements in the medium.

Although saturated fatty acids predominate in the 1 position (column I, Table I), unsaturated fatty acid chains, primarily cis-Δ^{11}-18:1, constitute about 30% of the residues. Studies characterizing the molecular species of an *E. coli* phosphatidylethanolamine indicate that those molecules carrying monoenoic acyl chains in the 1 position invariably have another unsaturated chain in the 2 position (Van Golde and Van Deenen, 1967). The extent to which unsaturated fatty acids are incorporated into the 1 position, then, may be taken as an indication of the frequency of molecules with two cis unsaturated residues.

The 2 position of phosphatidylethanolamine of wild-type cells (column II, Table I) is rich in unsaturated fatty acid residues, predominately cis-Δ^{9}-16:1, and the overall composition (column III, Table I) is about 60% unsaturated and about 40% saturated acyl residues.

In the various mutant cultures, the only cis unsaturated fatty acid consistently found to significant extents in the 1 position of phosphatidylethanolamine is cis-Δ^{11}-18:1 (culture 3, Table I). *cis*-Monoenoic fatty acids of shorter

[1] See legend to Table I.

TABLE I: Positional Distribution of Fatty Acids in Phosphatidylethanolamine Derived from Cells Supported on Fatty Acids of Normal Geometry.[a]

Strain and Fatty Acid Supplement	I 1 Position							II 2 Position									III Total							
	SFA				UFA			SFA					UFA				SFA				UFA			
	14	16	18	T	16	18	T	12	14	16	18	T	14	16	18	T	14	16	18	T	14	16	18	T
Wild type																								
1. Cis-Δ^{11}-18:1[b]	5	63	0	68	7	22	29	1	3	9	2	15	0	57	27	84	3	36	0	39	0	35	25	60
2. Cis-Δ^{9}-18:1	3	59	1	63	9	23	32	0	4	4	0	8	0	57	34	91	4	32	0	36	0	33	28	61
Mutant																								
3. Cis-Δ^{11}-18:1	8	71	4	83	0	16	16	0	11	13	0	24	0	18	58	76	11	43	0	54	0	13	32	45
4. Cis-Δ^{9}-16:1	3	88	4	95	0	0	0	0	6	17	2	25	0	75	0	75	4	54	0	58	0	38	0	38
5. Cis-Δ^{9}-16:1 plus cis-Δ^{11}-18:1	4	90	2	96	1	2	3	0	4	13	0	17	0	61	16	77	6	49	0	55	0	33	10	43
6. Cis-Δ^{9}-18:1	6	88	1	95	0	4	4	1	6	9	1	17	0	16	66	82	5	47	1	53	0	8	37	45
7. Cis-Δ^{9}-14:1	4	94	0	98	0	0	0	2	18	33	0	53	45	0	0	45	12	65	0	77	21	0	0	21

[a] All values are expressed as weight percentage of total fatty acids in each position or in the total phosphatidylethanolamine. Each value may vary ±2% between two separate runs with the same sample on the gas–liquid chromatograph. 1 position represents the fatty acids remaining on the lysophosphatidylethanolamine after treatment with phospholipase A as described in Methods; 2 position, the fatty acids released by the enzyme; total, the fatty acids derived from the phosphatidylethanolamine not treated with the enzyme. SFA, saturated fatty acids; UFA, unsaturated fatty acids or their cyclopropane derivatives; T, total saturated or unsaturated fatty acid in each position or in the total phosphatidylethanolamine. All conditions of supplementation were examined at least two different times. Data are presented in Tables I and II from cultures in which the composition *calculated* for the total phosphatidylethanolamine from that measured for the individual positions agreed very closely with that *determined directly* from the phosphatidylethanolamine not exposed to the enzyme. [b] The number before the colon gives the number of carbon atoms and the number after the colon gives the number of double bonds; superscript to Δ gives position of ethylenic bond.

TABLE II: Positional Distribution of Fatty Acids in Phosphatidylethanolamine Derived from Cells Supported on Fatty Acids of Abnormal Geometry.[a]

Strain and Fatty Acid Supplement	I 1 Position								II 2 Position									III Total								
	SFA					UFA			SFA					UFA				SFA					UFA			
	12	14	16	18	T	16	18	T	12	14	16	18	T	16	18	T	12	14	16	18	T	16	18	T
Wild type																								
1. Cis-Δ^{11}-18:1		5	63	0	68	7	22	29	1	3	9	2	15	57	27	84	0	3	36	0	39	35	25	60
Mutant																								
2. Trans-Δ^{11}-18:1		3	58	0	61	11	28	39	1	11	12	0	24	34	33	67	1	9	34	0	44	24	29	53
3. Trans-Δ^{9}-16:1	2	7	66	0	75	23	0	23	5	14	11	0	30	69	0	69	0	0	37	0	46	54	0	54
4. Cis-Δ^{6}-18:1		9	82	2	93	0	3	3	2	10	18	3	33	10	57	67	0	9	47	1	57	4	38	42

[a] See legend to Table I.

chain length than 18 carbons (cultures 4 and 7, Table I) or with ethylenic bonds closer to the carboxyl terminus than 11, 12 position (cultures 4, 6, and 7, Table I; culture 4, Table II) are less well, if at all, incorporated into the 1 position of the molecule. This observation is in keeping with the predominance of *cis*-Δ^{11}-octadecenoic over *cis*-Δ^{9}-hexadecenoic acid in the 1 position of phosphatidylethanolamine from the wild-type strain. Within the class of cis unsaturated fatty acids, acyltransferases acting at the 1 position select for longer chain length and for chains in which the ethylenic bond is furthest from the carboxyl terminus. It is not easy to test this hypothesis *in vivo* by mixed supplementation

as shown in culture 5 (Table I). When both cis-Δ^9-16:1 and cis-Δ^{11}-18:1 are provided as supplements in equal amounts, there is preferential utilization of cis-Δ^9-16:1 with the result that little cis-Δ^{11}-18:1 is assimilated into 1 position (or 2 position).

The major finding, then, in examining the fatty acid distribution of phosphatidylethanolamine from mutant cultures grown on cis unsaturated fatty acids of normal structure is a deficiency of unsaturated residues in the 1 position. As a result of this deficiency, mutant cultures supported on certain analogs do not fashion any phosphatidylethanolamine molecules containing two unsaturated chains.

The 2 position of phosphatidylethanolamine from mutant cultures contains predominately the unsaturated fatty acid supplement provided in the medium (column II, cultures 3–7, Table I). In addition, as noted in an earlier study (Silbert *et al.*, 1968), the mutant is able to derive and incorporate into the lipid small amounts of cis-16:1 residues through partial β oxidation of the cis-18:1 unsaturated acyl supplements (cultures 3 and 6, Table I). On the other hand, there is no elongation of cis-Δ^9-16:1 or cis-Δ^9-14:1 by the cell (cultures 4 and 7, Table I). These constitute the only unsaturated fatty acyl chains found in the 2 position. The proportion of saturated fatty acids found in the 2 position of mutant cultures is increased moderately relative to that present in phospholipid derived from wild-type cells. In mutant cultures where the incorporation of unsaturated chains is particularly low (culture 7, Table I), this appears to be counterbalanced by a greater per cent of tetradecanoic as well as hexadecanoic acid. Intermediate-chain-length saturated fatty acids in the 2 position may effect the physical properties of the phospholipid in a manner similar to cis unsaturated fatty acid residues (Van Deenen, 1967). The relative per cent of intermediate-chain-length acids, especially decanoic and dodecanoic acids, may be underestimated because of their volatility as esters and subsequent selective loss during analysis.

Positional Distribution of Fatty Acids in Phosphatidylethanolamine Derived from Cultures Supported on Analogs Structurally Unnatural for E. coli. The composition of the 1 position of phosphatidylethanolamine from mutant cells grown in the presence of *trans*-monoenoic supplements appears very similar to that of phosphatidylethanolamine from wild-type cells (compare column I, cultures 1 *vs.* 2 and 3, Table II). Gas–liquid chromatographic analysis does not discriminate well between geometrical isomers. However, earlier studies showed that there is not cis–trans isomerization during the assimilation of fatty acid supplements by the mutant strain (Silbert *et al.*, 1968). Therefore, the unsaturated fatty acid residues in the phosphatidylethanolamine of the mutant, in fact, are trans unsaturated fatty acids and have a geometry much more like saturated chains than like the cis unsaturated fatty acids present in the lipid of wild-type cells. The unsaturated fatty acid present in the 1 position of culture 3 (Table II) clearly reflects that provided in the medium; in culture 2 (Table II), some trans-16:1 arising by β oxidation is incorporated into the 1 position as well as into the 2 position. The moderate incorporation of trans-16:1 chains (column I, cultures 2 and 3, Table II) is in marked contrast to the virtual absence of cis-16:1 chains in the 1 position of mutant cultures (column I, cultures 3–6, Table I).

TABLE III: Relative Distribution of Unsaturated Fatty Acids between the 1 and 2 Positions of Phosphatidylethanolamine.[a]

Strain and Fatty Acid Supplement	Unsaturated Fatty Acid[b]	1 Position (%)	2 Position (%)	2 Position: 1 Position
Wild type				
1. Cis-Δ^{11}-18:1	16:1	11.3	88.7	7.8
	18:1	45.5	54.5	1.2
2. Cis-Δ^9-18:1	16:1	14.2	85.8	6.0
	18:1	39.8	60.2	1.5
Mutant				
3. Cis-Δ^{11}-18:1	16:1	0[c]	100.0	>100
	18:1	22.0	78.0	3.6
4. Cis-Δ^9-18:1	16:1	0	100.0	>100
	18:1	5.2	94.8	18.2
5. Cis-Δ^6-18:1	16:1	0	100.0	>100
	18:1	5.6	94.4	16.9
6. Cis-Δ^{11}-18:1 plus cis-Δ^9-16:1	16:1	1.1	98.9	90.0
	18:1	11.0	89.0	8.1
7. Cis-Δ^9-16:1	16:1	0	100.0	>100
8. Cis-Δ^9-14:1	14:1	0	100.0	>100
9. Trans-Δ^{11}-18:1	16:1	23.7	76.3	3.2
	18:1	46.3	53.7	1.2
10. Trans-Δ^9-16:1	16:1	25.0	75.0	3.0

[a] Data presented in Tables I and II recalculated to show specifically the positional distribution of unsaturated fatty acid residues. Growth rate of the auxotroph and total incorporation of supplement is not influenced by the concentration of the supplement in the medium; only the extent of growth is effected. [b] See legend to Table I. [c] 0% indicates that there was no measurable mass for the fatty acid at this position; that is, relative to the total composition of the position, less than 0.5%.

On the other hand, the incorporation data for the 2 position reveal that the trans fatty acids are incorporated more extensively than the long-chain saturated fatty acids. There is an increase of saturated fatty acids in the 2 position of phosphatidylethanolamine from cells supported on trans analogs compared with the levels found in the wild-type phospholipid, but this shift is due to increased incorporation of *intermediate-chain*-length saturated fatty acids.

It appears, then, that *trans*-monoenoic fatty acyl groups share structural features or physical properties in common with saturated fatty acyl chains in terms of acylation reactions involving the 1 position while at the same time they are more reactive than the *long-chain* saturated fatty acyl groups in the transacylations involving the 2 position.

The phosphatidylethanolamine derived from cells supported with trans unsaturated fatty acids is composed mostly of molecules containing two straight-long-chain acyl residues. This makeup is very atypical for naturally occurring phosphatides in which the major molecular species has one straight long chain in the 1 position but an acyl residue

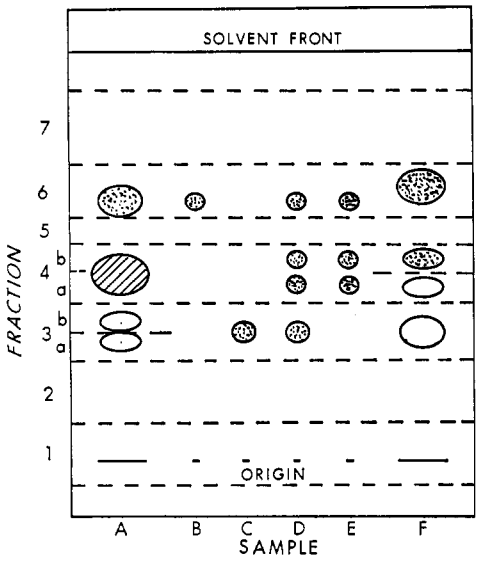

FIGURE 1: Thin-layer chromatographic separation of 1,2-diglycerides according to degree of unsaturation. Silica gel G–silver nitrate plate with chloroform–absolute ethanol (97:3, v/v) as solvent system. Details of procedure are described in Methods. (A) Diglycerides from phosphatidylethanolamine of unstarved cells; (B) 1,2-dipalmitin (R_F 0.58); (C) 1,2-diolein (R_F 0.34); (D) mixture of B, C, and E; (E) diglyceride from phosphatidylethanolamine of *E. coli* B containing predominantly diglycerides with monoenoic and saturated fatty acid residues per molecule (R_F 0.41 and 0.47) or with two saturated fatty acyl groups per molecule (R_F 0.58); and (F) diglycerides from phosphatidylethanolamine of starved cells. The relative amounts of diglyceride classes in the experimental samples as judged by fluorescence under ultraviolet light after spraying with 0.2% dichlorofluorescein: unshaded spots, minor component; stippled spots, moderate components; striped spots, major component.

TABLE IV: Distribution of Newly Synthesized Saturated Fatty Acids According to Diglyceride Species Derived from Phosphatidylethanolamine.[a]

Diglyceride Subfractions		Distribution of Radioactivity[d] (%)					
		Unstarved Cells			Starved Cells		
		1	2	3	1	2	3
	7[b]	1	0	0	2	1	2
SS[c]	6	20	31	24	86	85	83
	5	8	2	7	4	4	3
SU	4	57	48	45	3	4	4
UU	3	4	9	12	1	2	3
	1 plus 2	11	10	12	4	4	5

[a] At the time of preparation of the diglycerides, carrier phosphatidylethanolamine from *E. coli* B was added to the labeled phosphatide derived from fatty acid auxotroph. [b] Numbers correspond to fractions shown in Figure 1. [c] SS, diglyceride containing two saturated residues; SU, diglyceride esterified with one saturated and one unsaturated fatty acid; UU, diglyceride containing two unsaturated fatty acyl groups. [d] Diglyceride subfractions of phosphatidylglycerol, cardiolipin, and phosphatidylethanolamine derived from starved cells showed the same distribution of radioactivity.

in the 2 position which is commonly a cis unsaturated, a branched-chain, or an intermediate-chain-length (12 or 14 carbons) fatty acid (Van Deenen, 1967).

It is possible to change the makeup of the phosphatidylethanolamine from the auxotroph in yet another direction. When cis-Δ^6-18:1 is provided as supplement, it is incorporated in a pattern similar to cis-Δ^9-14:1 or cis-Δ^9-16:1, namely, almost exclusively into the 2 position (column II, culture 4, Table II). The predominant molecular species in this phosphatide class must contain a saturated chain on the 1 position and a cis-Δ^6-18:1 monoenoic acid on the 2 position. This type of molecule might have less apolar interactions between the chains on the 1 and 2 positions due to the proximity of the cis-ethylenic bond to the carboxyl terminus of the 2 substituent. Thus, molecules containing *cis*-Δ^6-octadecenoic acid are less compact while those carrying trans fatty acids are more compact than the normally predominant species in *E. coli* containing *cis*-Δ^9- and *cis*-Δ^{11}-octadecenoic acids (Van Deenen, 1967).

Positional Distribution of Unsaturated Fatty Acids. The unsaturated fatty acid contents of the 1 and 2 positions in phosphatidylethanolamine from the several cultures shown in Tables I and II have been recalculated (Table III) to show specifically the relative positional affinities of the different monoenoic fatty acids. The distributions are subject to the influence of the amounts and kinds of endogenously produced fatty acyl thio esters. However, the pattern seen in Tables I and II comes out more clearly here: among *cis*-monoenoic fatty acids, chain length and position of the ethylenic bond relative to the carboxyl terminus significantly influences incorporation into the 1 position; for any given pair of geometrical isomers used as supplements in this study, the trans analog is incorporated more extensively into the 1 position than its cis counterpart. Also, chain length (or possibly location of the double bond) of the trans unsaturated fatty acid has less of an effect on the distribution of the residue than in the case of the cis isomer.

Fatty Acid Starvation. A second approach to altering the acyl chain character of the newly synthesized phospholipid involves short-term starvation during which time the organism continues to grow at normal rates. The effect of this manipulation is the cessation of synthesis of phospholipid molecules containing either two or one cis unsaturated acyl groups. In order to establish this point the following experiment was performed. The fatty acid auxotrophs can incorporate [^{14}C]acetate into saturated fatty acids but not into unsaturated fatty acids because of the nature of the mutation and absence of elongation of exogenous supplements. Thus, the relative synthesis of the molecular types containing one or two saturated fatty acid chains can be followed in response to unsaturated fatty acid starvation by determining the distribution of [^{14}C]acetate into these two types of molecules. The saturated acyl groups of the phospholipids of starved and supplemented cells were labeled under conditions described in the Methods section and the individual phospholipids were isolated. The various molecular species were then separated according to the methods of Van Golde and Van Deenen (1967) after the phospholipid had been converted

TABLE V: Fatty Acid Composition of the Diglyceride Subfractions Derived from Phosphatidylethanolamine.[a]

Diglyceride Subfraction	Fatty Acid Composition (mole %)							Total in Diglyceride Subfraction
	SFA				UFA			
	14	16	18	Total	16	18	Total	
Unstarved cells[b]								
SS[d]	3.0	18.4	0.4		0.1	1.2		23.1
SU	1.9	30.8	0.4		6.1	26.4		65.6
UU	0.0	0.8	0.0		2.5	8.0		11.3
Sum	4.9	50.0	0.8	55.7	8.7	35.6	44.3	100.0
Starved cells[c]								
SS	17.7	43.8	0.7		0.0	0.7		62.9
SU	1.3	18.3	0.0		3.0	12.0		34.6
UU	0.0	0.3	0.0		0.3	1.9		2.5
Sum	19.0	62.4	0.7	82.1	3.3	14.6	17.9	100.0

[a] The preparation and fractionation of diglyceride and the characterization of their fatty acid composition are described in the Experimental Section. At the time that the cells were harvested, the yield of phosphatidylethanolamine from the starved cells was approximately 15% lower than that from the unstarved cells. [b] Based on their individual fatty acid compositions, the data from fractions shown in Figure 1 for the unstarved preparation were combined as follows: (UU) — F_x3a containing 100% and F_x3b containing more than 90% unsaturated chains; (SU) — F_x4 containing equal amounts of the two classes of fatty acids; (SS) — F_x5 containing more than 90% and F_x6 and F_x7 containing 100% saturated residues. [c] The data from the fractions shown in Figure 1 for the starved sample were pooled as follows; (UU) — F_x3 containing about 90% unsaturated fatty acids; (SU) — F_x4a containing equal amounts of saturated and unsaturated (about 75% 16:1 and 25% 18:1) chains and F_x4b also containing nearly equal quantities of saturated and unsaturated (96% 18:1 and 4% 16:1) fatty acids; (SS) — F_x5 containing more than 90% and F_x6 containing 100% saturated residues. [d] See legend to Table IV for explanation of abbreviations.

into the free 1,2-diglycerides by treatment with phospholipase C. Fractionation of the diglycerides on a silica gel–silver nitrate plate is shown schematically in Figure 1.

Among the diglyceride subfractions derived from unstarved cells, the major radioactive component is the diglyceride containing one saturated chain (Table IV). This result is consistent with the observation that this species alone normally accounts for more than 50% of the molecules (Table V). Under conditions of starvation, the pattern of incorporation is shifted so that virtually all the radioactivity now migrates with molecules containing two saturated acyl residues. This finding also holds for the distribution of [^{14}C]acetate label among diglyceride subfractions derived from phosphatidylglycerol or cardiolipin isolated from starved cells.

In order to show more directly the pattern of lipid synthesis under these starvation conditions, the composition of the various diglyceride fractions were examined by gas–liquid chromatography (Table V). The data confirm the apparent fractionation seen in Figure 1. That is, there is excellent separation of molecules containing two saturated or two unsaturated chains from a fraction containing equal amounts of each class of fatty acid. Comparing the two culture conditions, for every 100 molecules in the unstarved culture, there are 23, 66, and 11 molecules containing, respectively, two, one, or no saturated fatty acid residues (last vertical column, Table V); in the starved culture, the distribution shifts to 63, 35, and 2 molecules for the same molecular types, respectively. Since the cells underwent approximately one doubling, there were initially 50 molecules distributed in the proportions 12, 32, and 6 for the three different subfractions containing 2, 1, or no saturated fatty acid chains. The change to 63, 35, and 2 for these same subfractions during the doubling of cells mass represents a 5-fold increase in molecules with only saturated fatty acids, no increment in those containg one saturated and one unsaturated group, and a threefold reduction in the preexisting molecules with two unsaturated fatty acid residues. These observations are borne out by the changes in overall and individual fatty acid compositions associated with starvation.

The overall composition shifts from 56% saturated and 44% unsaturated fatty acids to 82% saturated and 18% unsaturated residues in the phosphatidylethanolamine of the starved cells. This change in saturated chains is due to a very large incorporation of tetradecanoic acid as molecules with two saturated chains are formed. The mole per cent of tetradecanoic acid increased over the period of starvation from 3.0 to 17.7% in the diglyceride subfraction containing only saturated chains. The increment in the other saturated chains is much less marked. Consistent with the cessation of synthesis of molecules containing unsaturated chains, there is a 2-fold decrement in the mole per cent of the individual fatty acids composing the SU[2] molecules. The greater decrease the UU[2] molecules cannot be attributed solely to

[2] See legend to Table IV.

TABLE VI: Specific Radioactivity of Saturated Fatty Acids in Diglycerides Derived from Phosphatidylethanolamine.[a]

Diglyceride Subfraction	Sp Radioactivity of Satd Fatty Acid (mμCi/μg)	
	14	16
Unstarved cells		
SS[b]	1.58	1.77
SU		1.72
Starved cells		
SS	2.56	1.74
SU		0.17

[a] Diglyceride subfractions corresponding to the separation shown in Figure 1 were individually analyzed for the radioactivity and mass associated with the saturated fatty acids as described under Methods. For each specific radioactivity, the results of several determinations varied within 10% of the average value which is the number given in the table. [b] See legend to Table IV for explanation of abbreviations.

dilution and suggests that there may be active turnover of this type of molecule under starvation conditions.

In Table VI, the specific radioactivity of the saturated fatty acid chains (expressed as millimicrocurie per microgram to correct for molecular weight differences) in the diglyceride subfraction of phosphatidylethanolamine is presented. The near equivalence of all the specific radioactivities determined for the fatty acids derived from the unstarved cells demonstrates that the differential rate of synthesis during the period of labeling (and for several generations before) remains constant for the two types of molecules containing saturated fatty acids and for the two different chain lengths present in one of these diglyceride subfractions (SS).[2] In the starved culture, the specific radioactivity of hexadecanoic acid is 10-fold lower in molecules containing one unsaturated and one saturated fatty acyl group compared to those carrying two saturated chains. The specific radioactivity of tetradecanoic acid is 1.5-fold higher than that of hexadecanoic acid suggesting a greater differential rate of synthesis and incorporation into phospholipid.

The very much lower specific radioactivity of the diglyceride containing one unsaturated chain is direct evidence for the cessation of synthesis of this type of molecule under conditions of starvation.

The mass data in Table V indicated that during starvation the mole per cent of tetradecanoic and hexadecanoic acids in the SS subfraction increased 6- and 2-fold, respectively. One might expect the specific radioactivity of tetradecanoic to be 3-fold greater than that of hexadecanoic acid derived from the same diglyceride subfraction in the starved cells. The finding that it is only 1.5 times higher suggests that there is a source of unlabeled tetradecanoic acid in the culture. Small amounts of free tetradecanoic and hexadecanoic acids are introduced into the culture medium as contaminants of the Tween 40 detergent. Under conditions of starvation, these fatty acids may be assimilated into the phospholipid, tetradecanoic more than hexadecanoic acid. In the supplemented culture, preferential activation and incorporation of *cis*-Δ^9-octadecanoic acid into the phospholipid may effectively eliminate utilization of the exogenous saturated fatty acids. This mechanism might also explain why the specific radioactivities of tetradecanoic and hexadecanoic acids were not 6-fold and 2-fold higher, respectively, in the SS subfraction of the starved compared with the unstarved cells.

Discussion

The principal goal of this study was to characterize the arrangements of fatty acyl groups in the major phospholipid derived from an unsaturated fatty acid auxotroph supplemented with various fatty acid analogs or subject to deprivation of any unsaturated fatty acid supplement. The positional distribution of the acyl chains of phosphatidylethanolamine from the auxotroph used in the present study appears to be very sensitive to the type of exogenous supplement, particularly with respect to the 1 position. In addition, if exogenous fatty acids are transferred to phospholipid *via* coenzyme A thio esters (Silbert *et al.*, 1968) but fatty acyl groups synthesized *de novo* are donated from acyl-carrier protein thio ester, then a preference for the latter could influence the degree of incorporation of exogenous unsaturated fatty acids. For example, it might account for the lower levels of *cis*-Δ^{11}-octadecanoic and *cis*-Δ^9-hexadecanoic acids found in the 1 position of phospholipid from the unsaturated fatty acid auxotroph (cultures 3 and 4, Table I and cultures 3 and 7, Table III) as compared with that present in the phospholipid of the prototrophic organism (cultures 1 and 2, Tables I and III).

Where the diglyceride subfractions were determined for the phosphatidylethanolamine of the auxotroph supported on *cis*-Δ^9-octadecanoic acid, 11, 66, and 23 of the molecules contained, respectively, 2, 1, or no monoenoic chains. These results may be compared with those of Van Golde and Van Deenen (1967) for an extracellular phosphatidylethanolamine released by a lysine-requiring strain of *E. coli:* the relative proportion of the same diglyceride subfractions were, respectively, 20, 68, and 10. The frequency of different molecular species are normally probably adjusted to the environmental temperature. The wild-type cell incorporates increasing amounts of *cis*-Δ^{11}-octadecanoic acid into its phospholipid in response to decreasing temperatures as shown by Marr and Ingraham (1962). It seems likely that this change is reflected in an increased proportion of molecules containing two unsaturated fatty acids. The unsaturated fatty auxotroph supported on *cis*-monoenoic acids shorter than 18 carbons, already deficient at 37° in this type of molecular species, might not be able to fashion lipids suitable at lower temperatures for certain membrane associated functions. A recent study by Esfahani *et al.* (1969) indicates that the total incorporation of *cis*-Δ^9-hexadecanoic and *cis*-Δ^{11}-octadecanoic acids by an unsaturated fatty acid auxotroph increases as the temperature of growth is decreased from 42 to 27°. The extent of incorporation of the hexadecanoic acid was significantly less than that of the octadecanoic acid at all temperatures. The positional distribution of unsaturated residues in response to temperature was not examined. It would be of some interest to determine how much of the

increased incorporation of each of the supplements at lower temperature reflected the synthesis of molecules containing two unsaturated chains.

When trans unsaturated fatty acids were used as supplements, there was extensive incorporation into the 1 and 2 positions. The incorporation into the 1 position are consistent with the observations made by many others that the physical properties of trans unsaturated resemble saturated chains (Chapman, 1966) and that acyl transferases acting on the 1 position do not discriminate well between a trans unsaturated and a saturated fatty acid (Lands, 1964; Selinger and Holman, 1965; McElhaney and Tourtellotte, 1970).

With the exception of those molecules containing intermediate-chain saturated fatty acids, there is essentially only one type of molecule *with respect to physical properties* in the phospholipid of the mutant grown on trans unsaturated fatty acids: that is, a molecule containing two straight-long-chain fatty acyl groups. In studies of their morphology and functional properties previously discussed (Silbert *et al.*, 1968) or to be described more extensively elsewhere (D. F. Silbert, F. Ruch, J. Williamson, and P. R. Vagelos, unpublished data), cultures supported on trans unsaturated fatty acids have precarious survival capacity: growth is delayed, slow, and often incomplete. The cells are difficult to subculture and tend to form long filaments due to a failure to form septa. In addition, Esfahani *et al.* (1969) have reported that cells supplemented with these analogs lyse on lowering the temperature from 35 to 27°. These several observations appear to correlate well with the surface properties of pure phospholipids containing *trans*-monoenoic fatty acid (Chapman *et al.*, 1966). The degree of movement of the acyl chains in molecules containing trans unsaturated residues is very sensitive to the temperature range associated with function in biological systems. While molecules containing cis unsaturated groups form an expanded monolayer at an air–water interface at 20°, molecules containing the trans analogs give expanded monolayers only at higher temperatures, such as 34°. The possible biological relevance of these physical properties is supported by Van Deenen's observation (1967) that the predominant molecular species of all naturally occurring phospholipids forms expanded monolayers at 20°.

Starvation of the auxotroph for unsaturated fatty acids appears to acutely precipitate the synthesis of molecules physically equivalent to those found in the cells supported on trans analogs. This shift is due to an increased synthesis and incorporation into phospholipid of saturated fatty acids as seen here with a fatty acid auxotroph and also by Kass (1968) following the administration to prototrophic cells of a selective inhibitor of unsaturated fatty acid synthesis. The total incorporation of hexadecanoic acid into phosphatidylethanolamine appears to be only slightly increased but the increase is virtually all distributed to the molecules containing only saturated residues. The major increase in saturated chains is associated with an increased incorporation of tetradecanoic acid. The positional distribution of these saturated fatty acids remains to be shown.

Fox (1969) has shown that the induction of or maintenance of preexisting active lactose permease, a membrane protein, does not occur during the period of short-term unsaturated fatty acid starvation, although translation of the lactose operon continues as evidenced by the formation of galactoside acetylase as well as galactosidase. Restoration of cis unsaturated fatty acid supplements allows the preservation of residual preexisting transport activity. Under starvation conditions we see the cessation of synthesis of molecules containing one or two cis unsaturated fatty acids with a possible turnover of preexisting molecules, particularly those containing two unsaturated chains; also, there is an apparent shift in the pattern of glycerophosphatide synthesis favoring the accumulation of cardiolipin (Henning *et al.*, 1969). The activity of the lactose permease appears to require the continued synthesis of molecular species containing cis unsaturated chains. How specific this requirement is for a molecular type with one *vs.* two cis unsaturated chains or for a given glycerophosphatide class has yet to be defined.

Recent work of Van den Bosch and Vagelos (1970) strongly suggests that the selectivity for acyl donor at the level of lysophosphatidic and phosphatidic acid synthesis in *E. coli* could well account for the pattern of incorporation of fatty acids into phospholipid observed *in vivo* with the fatty acid auxotroph both under conditions of analog supplementation and fatty acid starvation. Work in progress to characterize the positional distribution of acyl groups in phosphatidylglycerol and cardiolipin when compared with that in phosphatidylethanolamine should indicate whether or not synthesis of the common precursor, phosphatidic acid, alone determines the ultimate distribution of acyl groups in the glycerophosphatides of *E. coli*.

The fatty acid composition of *Mycoplasma laidlawii*, in contrast to *E. coli*, can be influenced extensively by the concentration and type of fatty acid provided in the medium. The relative positional affinities of a large variety of saturated and unsaturated fatty acids in the major polar lipid, phosphatidylglycerol, has been examined by McElhaney and Tourtellotte (1970). The present observations on positional distribution of fatty acids in the *E. coli* phospholipids are in excellent agreement with the *Mycoplasma* studies, although the same degree of control by exogenous supplementation is not yet possible for the *E. coli* system.

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Nucleotide Clusters in Deoxyribonucleic Acids. Separation of Oligonucleotides Released by Deoxyribonuclease I*

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ABSTRACT: Oligonucleotides released from DNA by pancreatic deoxyribonuclease have been separated according to chain length up to the pentanucleotide level, by chromatography on DEAE-Sephadex at 65° using LiCl in 0.005 M Tris-HCl buffer (pH 7.6), 7 M in urea, as the eluting salt. The method is quantitative and good resolution of the longer isostichs has been obtained. All possible nonisomeric dinucleotides and 14 of the 20 possible nonisomeric trinucleotides have been separated quantitatively according to base composition by chromatography on DEAE-cellulose at pH 3.4 and 3.6, respectively, using ammonium formate as the eluting salt.

The 16 possible dinucleotides were resolved into 11 fractions and the proportions of the unresolved pairs of positional isomers determined by degradative analysis. The distribution of oligonucleotides isolated from a manganese-activated pancreatic deoxyribonuclease hydrolysate of *Escherichia coli* DNA was nonrandom. In the total dinucleotide fraction guanine and cytosine predominated at both the 3′ and 5′ ends. In dinucleotides which contained adenine and thymine, adenine and thymine predominated at the 3′ end. These results, compared to similar studies on magnesium-activated hydrolysis, support the possibility of a different specificity of degradation according to the activating ion used.

Studies on oligonucleotides released by enzymatic digestion of DNA have been limited by a lack of separation methods sensitive enough to produce discrete resolution of the complex mixtures of oligonucleotides. Chromatographic resolution is poor unless secondary binding forces, particularly those due to purines, are overcome. Tomlinson and Tener (1963) introduced the use of 7 M urea to reduce secondary binding forces in ion-exchange chromatography of polynucleotides on DEAE-cellulose columns. This modification was extended to DEAE-Sephadex chromatography by Rushizky *et al.* (1964). These methods have been used widely for fractionation of RNase and DNase digests but resolution above the octanucleotide level is poor (Carrara and Bernardi, 1968; Niyogi, 1969; Nestle and Roberts, 1969). Sedat and Sinsheimer (1964) used DEAE-Sephadex and 7 M urea in a heated column in order to fractionate purine oligodeoxynucleotides released by chemical degradation of DNA and achieved a separation according to chain length up to the dodecanucleotide level.

Fractionation of oligonucleotides according to base composition has been attempted by various techniques including ion-exchange (Vanecko and Laskowski, 1961; Becking and Hurst, 1963; Rushizky *et al.*, 1965; Solymosy

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