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# Utilization of Exogenous Fatty Acids for Complex Lipid Biosynthesis and Its Effect on *de Novo* Fatty Acid Formation in *Escherichia coli* K-12†

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ABSTRACT: The utilization of exogenous fatty acids by wild-type *Escherichia coli* K-12 has been studied using [$^{14}$C]acetate incorporation to monitor fatty acid synthesis and unusual fatty acid analogs as a nonradioactive supplement to distinguish acyl groups in the phospholipid derived from the exogenous source. Certain strains are found to regulate synthesis of saturated and unsaturated fatty acids in response to exogenous fatty acid supplements. This effect is associated with incorporation of the exogenous supplement into phospholipid. Even and odd chain-length saturated, trans and cis unsaturated fatty acids were included among the fatty acid supplements examined. Although a principal result of the incorporation of the exogenous fatty acid is a replacement of structurally related acyl groups, the effect is pleotropic in many instances: for example, 16:0 replaces 18:1 and *vise versa*. These observations can be rationalized in terms of the positional distribution in the phospholipid of the acyl residues derived from synthesis and the pattern of incorporation of the exogenous fatty acids. β-OH-14:0 and -12:0, added to cultures as supplements, are not detectably incorporated into complex lipid (would be predominantly lipid A) and show little or no influence on fatty acid synthesis.

Recent observations (Esfahani *et al.*, 1971; Sinensky, 1971; Silbert *et al.*, 1972) have demonstrated that certain strains of *Escherichia coli* K-12 are capable of regulating unsaturated fatty acid biosynthesis in response to an unsaturated fatty acid supplement in the medium. When *cis*-$\Delta^9$-octadecenoate is added to the culture, *cis*-$\Delta^{11}$-octadecenoate and *cis*-$\Delta^9$-hexadecenoate synthesis are markedly reduced together with a small decrease in the production of tetradecanoate and hexadecanoate (Silbert *et al.*, 1972). It is thought that the specificity of the response may reflect the extent to which the exogenous unsaturated fatty acid can replace various endogenously derived fatty acyl groups in the complex lipids (Silbert *et al*,, 1972). In the present study saturated, cis and trans unsaturated, and β-hydroxy fatty acids are tested as supplements in exponentially growing cultures of the two strains which had demonstrated regulation of unsaturated fatty acid synthesis in the previous study. The results clearly show a relationship between the regulation of fatty acid synthesis by exogenous fatty acids and the fashion and extent to which the external fatty acids are incorporated into phospholipid.

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## Materials and Methods

*Bacterial Strains.* All bacterial strains used in this study are *E. coli* K-12 strains. AB1623 and K1060 (the latter is defective in fatty acid degradation and in unsaturated fatty acid biosynthesis) have been described previously (Silbert *et al.*, 1972). K19, obtained from P. Overath, has the same genotype as K1060 without the fatty acid biosynthetic defect and without an unknown mutation facilitating growth of K1060 on trans unsaturated fatty acid supplement. AB1623-1 was derived by conjugation of AB1623 with HfrC *oldE* (a gift of J. Cronan). The simultaneous inheritance of arabinose and lactose fermentation from HfrC *oldE* was used to select for the transfer of the *oldE* gene. A suitable test for fatty acid degradation by AB1623 is the conversion of *trans*-$\Delta^{11}$-18:1 to *trans*-$\Delta^{9}$-16:1 and the incorporation of both fatty acids in almost equal amounts into phospholipid when *trans*-$\Delta^{11}$-18:1 is provided as a growth supplement. AB1623-1 is an *ara*$^+$ *lac*$^+$ derivative of AB1623 which incorporates *trans*-$\Delta^{11}$-18:1 into phospholipid but does not convert it to *trans*-$\Delta^{9}$-16:1 as evidenced by its absence from the phospholipid.

*Chemicals.* The fatty acids, except for $\beta$-hydroxymyristate, were obtained as >99% pure from Hormel Institute, Austin, Minn., DL-$\beta$-hydroxymyristate was the gift of Dr. Robert Bell, D(−)-$\beta$-hydroxymyristate was isolated from *E. coli* K-12 by means of acid hydrolysis of cell pellet, ether extraction, and silicic acid column chromatography according to the procedure of Preiss and Bloch (1964) and was 99% pure as judged by thin-layer and gas–liquid chromatography. Brij 58 (poly(oxyethylene cetyl ether)) was obtained from Atlas Chemical Co., Wilmington, Del.; [1-$^{14}$C]acetate was a product of New England Nuclear Corp., Boston, Mass. To remove contaminating lipids, reagent grade chloroform and methanol were redistilled over potassium permanganate and potassium carbonate before use. Mallinckrodt anhydrous ethyl ether and nanograde *n*-pentane were suitable for use without purification.

*Radioisotope Labeling.* AB1623 cells, grown overnight in medium 63 (Pardee *et al.*, 1959) containing 0.4% glycerol, 5 mM potassium glutamate, 0.00005% thiamine, 0.00005% yeast extract, 0.4 mM L-leucine, 1.25 mM L-isoleucine, 1.0 mM L-valine, 1 mM potassium acetate, and 0.1% Brij 58, were collected by centrifugation, washed with medium 63, and resuspended at about $5 \times 10^7$ cells/ml in fresh medium containing the above ingredients. The cell suspension was subdivided into 25-ml portions to which fatty acid supplements were added at 50 $\mu$g/ml (about 0.2 mM) for each fatty acid and then all the cultures were placed in a gyrotory shaker at 37°. After 1.5 generations, when logarithmic growth had resumed, 10 $\mu$Ci of [1-$^{14}$C]acetate was added accurately to each culture, exponential growth was followed for 2.5–3.0 generations, and then the cells were harvested at room temperature by centrifugation at 35,000*g*, washed once with 0.1 M potassium phosphate (pH 7.0) and stored at −20° until subjected to acid hydrolysis as discussed below. As shown in Table I, all cultures grew with very nearly identical rates (1.3–1.4 hr/generation) and were labeled with [$^{14}$C]acetate over the same cell densities for nearly identical periods of time (2.5–2.9 generations). K1060 cells were handled in an identical fashion except that the basic growth medium consisted of medium 63 containing 0.4% glycerol, 0.00005% thiamine, 0.1% Brij 58, 0.005% potassium oleate, and 1 mM potassium acetate. All cultures grew with 1.6- to 1.8-hr generation times and were labeled for 2.6–2.9 generations (see Table I). The specific radioactivities of the major fatty acids derived from these cultures are also shown in Table I. The methods for extracting the fatty acid and measuring the specific radioactivities are described below. The values obtained show that the specific radioactivity of the same fatty acid derived from unsupplemented or supplemented cultures of the same strain (labeled in parallel as described above) is constant within experimental error in many cases (*e.g.*, Table I, compare 12:0 in cultures 1, 2, 3; 14:0 in 6, 7, 8, 9; 16:0 in 1 and 2 or in 6 and 8; 16:1 and 18:1 in 1 and 3; $\beta$-OH-14:0 in 1, 2, 3 or in 6, 7, 8, 9). The specific radioactivity of the precursor acetate within the cell must be essentially the same in the different cultures of a given strain and, therefore, the incorporation of [$^{14}$C]acetate into fatty acid is a reliable measure of the extent of synthesis of that fatty acid in the different cultures. A lower specific radioactivity for a particular fatty acid in supplemented cultures relative to that in unsupplemented AB1623 or in 18:1 supplemented K1060 cultures is also observed in several cases (*e.g.*, Table I, compare 14:0 in cultures 3 *vs.* 1; 16:0 in 3 *vs.* 1 or in 7 and 9 *vs.* 6; 16:1 and 18:1 in 2 *vs.* 1). We attribute this effect (1) to dilution, that is, the fatty acid is derived in part either directly or by $\beta$-oxidation (applies to AB1623 but not to K1060 cells which are blocked by mutation in $\beta$-oxidation) from the unlabeled supplement provided in the medium; or (2) to regulation, that is, its synthesis is reduced during the [$^{14}$C]acetate pulse period in response to the supplement.

The extent of synthesis of *different* fatty acids within a *given* culture is also reflected in the specific radioactivities shown in Table I. In particular, the specific radioactivity of $\beta$-hydroxymyristate is consistently higher and that of 12:0 is sometimes higher (*e.g.*, Table I, cultures 6 and 8) when referred to that of 16:0 in the same culture. These differences indicate that the synthesis or turnover of these chains (12:0 and $\beta$-OH-14:0) is not coordinate with that of the others during the period of labeling. These fatty acids are major constituents of lipid A, and the higher specific radioactivity may reflect turnover due to release of lipid A containing structures into the medium as previously observed (Knox *et al.*, 1967; Rothfield and Pearlman-Kothenz, 1969; Silbert *et al.*, 1972).

*Isolation and Analysis of Fatty Acids from Cells Labeled with Radioisotopes.* Procedures for handling the cells grown in the presence of [$^{14}$C]acetate and for analysis of the cellular lipids have been previously described (Silbert *et al.*, 1972) and include acid hydrolysis of the whole cell pellet, extraction and conversion of the fatty acids to methyl esters, and the analysis of radioactivity in (1) saturated, unsaturated and hydroxy fatty acid classes by thin-layer chromatography on silica gel G containing silver nitrate followed by liquid scintillation counting of the methyl ester fraction and in (2) individual fatty acids by coupled gas–liquid gas radiochromatography. The mass response produced by the fatty acid esters emerging from the gas–liquid chromatograph was quantitated by an Infotronics digital readout system, Model CRS-100 with the results subject to approximately 3% error (Janak, 1960). The radioactivity associated with each fatty acid peak was computed from the digital integrator readout of the gas radiochromatograph. As discussed elsewhere (Silbert *et al.*, 1972), a major source of error in this measurement arises from fluctuations in the background radioactivity. The standard deviation for the background counts under each peak was determined and this value taken as the measure of error. The background radioactivity equalled 20% or more of the gross counts, increasing inversely with the size of the radioactive peak. Specific radioactivities were obtained after calibrating the responses of the gas–liquid and gas radiochromatographs with appropriate standards and the error was calculated by sum-

TABLE I: Properties of Radioisotopically Labeled Cultures.[a]

| Culture | Supplement | Generation Time (hr) | Generations Labeled | Sp Radioactivity (nanocuries/μg)[a] SFA[b] 12:0 | SFA[b] 14:0 | SFA[b] 16:0 | UFA 16:1 | UFA 18:1 | UFA ?[c] | HFA βH-14:0 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. AB1623 | None | 1.4 | 2.5 | 2.4 (±0.4) | 1.5 | 2.0 | 2.0 | 2.0 (±0.6) | 2.0 (±0.6) | 2.8 (±0.6) |
| 2. AB1623 | 18:1[d] (U)[b] | 1.3 | 2.9 | 1.9 | 1.4 | 1.8 | 1.0 | 0.2 | 0.0 | 2.7 |
| 3. AB1623 | 16:0 | 1.6 | 2.6 | 1.8 | 1.0 | 0.3 | 1.7 | 1.6 (±0.5) | 1.7 (±0.7) | 2.3 |
| 4. AB1623 | β-OH-14:0 (H) | 1.3 | 3.0 | | | Not determined | | | | |
| 5. AB1623 | 18:1, 12:0, 14:0, 16:0, β-OH-14:0 (USH) | 1.4 | 2.6 | | | Not determined | | | | |
| 6. K1060 | 18:1 (U) | 1.6 | 3.2 | 4.2 | 3.1 | 3.2 | 0.0 | 0.0 | 0.0 | 5.0 |
| 7. K1060 | 18:1, 16:0 (US) | 1.8 | 2.9 | 3.5 | 2.7 | 0.2 (±0.04) | 0.0 | 0.0 | 0.0 | 4.5 |
| 8. K1060 | 18:1, β-OH-14:0 (UH) | 1.7 | 2.7 | 3.8 | 3.1 | 3.0 | 0.0 | 0.0 | 0.0 | 4.4 |
| 9. K1060 | 18:1, 16:0, β-OH-14:0 (USH) | 1.7 | 2.9 | 2.8 | 2.8 (±0.5) | 0.1 (±0.04) | 0.0 | 0.0 | 0.0 | 4.6 |
| 10. K1060[e] | 18:1, 12:0, 14:0, 16:0, β-OH-14:0 (USH) | 2.3 | 2.1 | | | Not determined | | | | |

[a] The values for each culture were derived from the data of a single gas liquid chromatography gas radiochromatography coupled run, analyzing an aliquot of the total fatty acid methyl esters obtained from the cells. 0.0 means that there is no significant radioactivity in this fraction, that is, the error of the measurement is equal or greater than the number measured. The number in parentheses below the specific radioactivity is the error estimate (see Methods). If none is shown, the following percentage errors apply: 12:0, 14:0, and β-OH-14:0, less than 15%; 16:0 and 16:1, less than 10%; 18:1 and ?, less than 25%. [b] SFA or S, saturated fatty acid(s); UFA or U, unsaturated fatty acid(s); HFA or H, hydroxy fatty acid(s). [c] Arises from cyclopropane derivatives of unsaturated fatty acid. See Silbert *et al.* (1972) and text. [d] 18:1 used as supplement is *cis*-$\Delta^9$-18:1. [e] This culture was grown in a separate experiment in which the control 18:1 supplemented culture had a generation time of 1.9 hr and was labeled for 2.8 generations. Growth of the multiply-supplemented culture 10 began to slow down over the last 0.5 generation.

TABLE III: Amounts (nanocuries/mg of protein) of Individual Fatty Acids Synthesized.[a]

| Culture[c] | Supplement | Saturated Fatty Acids 12:0 | 14:0 | 16:0 | Total | Unsaturated Fatty Acids 16:0 | 18:1 | ? | Total | HFA β-OH-14:0 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. AB1623 | None | 5.2 (100) | 17.9 (100) | 57.4 (100) | 81.5 (100) | 33.8 (100) | 5.9 (100) | 5.4 (100) | 45.1 (100) | 26.0 (100) |
| 2. AB1623 | 18:1[d] (U)[b] | 5.0 (96) | 14.8 (83) | 39.0 (68) | 58.8 (72) | 7.6 (22) | 2.9 (47) | 0.0 (0) | 10.5 (23) | 22.6 (87) |
| 3. AB1623 | 16:0 (S) | 7.3 (141) | 13.8 (77) | 17.0 (30) | 38.1 (47) | 31.8 (94) | 3.2 (54) | 4.8 (89) | 39.8 (88) | 30.4 (117) |
| 6. K1060 | 18:1 (U) | 3.3 (100) | 5.6 (100) | 17.3 (100) | 26.9 (100) | 0.0 | 0.0 | 0.0 | 0.0 | 11.1 (100) |
| 7. K1060 | 18:1, 16:0 (US) | 5.4 (164) | 3.7 (66) | 2.0 (12) | 11.5 (43) | 0.0 | 0.0 | 0.0 | 0.0 | 12.2 (100) |
| 8. K1060 | 18:1, β-OH-14:0 (UH) | 3.2 (97) | 5.6 (100) | 19.2 (111) | 29.1 (108) | 0.0 | 0.0 | 0.0 | 0.0 | 11.6 (104) |
| 9. K1060 | 18:1, 16:0, β-OH-14:0 (USH) | 5.8 (176) | 3.6 (64) | 1.8 (10) | 11.2 (42) | 0.0 | 0.0 | 0.0 | 0.0 | 12.4 (112) |

[a] Nanocuries per milligram were calculated by multiplying the total fatty acid radioactivity obtained from the cell per milligram of total cell protein times the fraction of recovered radioactivity found in each fatty acid ester after a single analytical gas liquid chromatographic gas radiochromatographic run on an aliquot of the fatty acid esters derived from the cells. See legend to Table I for definition of 0.0 and the error estimates for specific fatty acids (compounded by summing the per cent error in measuring radioactivity and the per cent error in determining protein) which also apply here. Exceptions to this rule are 19 and 17% for 12:0 in cultures 1 and 7, respectively; 20 and 39% for 16:0 in cultures 7 and 9, respectively; 12% for 16:0 in culture 2; 32 and 34% for 18:1 and 33 and 44% for ? in cultures 1 and 3, respectively; 23% for β-OH-14:1 in culture 1. The numbers in parentheses express the nanocuries per milligram obtained for a given fatty acid in a given culture as a percentage of the value measured for the same fatty acid in the control culture (culture 1 for AB1623 and culture 6 for K1060). [b] See legend to Table I. [c] See legend to Table I. [d] See legend to Table I. [e] See legend to Table II.

TABLE II: Amounts (nanocuries/milligram of protein) of Saturated, Unsaturated, and Hydroxy Fatty Acids Synthesized.[a]

| Culture[e] | Supplement | Saturated Fatty Acids | Unsaturated Fatty Acids | Hydroxy Fatty Acids |
|---|---|---|---|---|
| 1. AB1623 | None | 87.6 (100) | 41.3 (100) | 24.6 (100) |
| 2. AB1623 | 18:1[d] (U)[b] | 63.0 (72) | 10.8 (26) | 19.6 (80) |
| 3. AB1623 | 16:0 (S) | 47.0 (54) | 35.6 (86) | 26.6 (108) |
| 4. AB1623 | β-OH-14:0[c] (H) | 80.1 (91) | 44.2 (107) | 21.1 (86) |
| 5. AB1623 | 18:1, 12:0, 14:0, 16:0, β-OH-14:0 (USH) | 47.7 (54) | 36.0 (87) | 19.8 (80) |
| 6. K1060 | 18:1 (U) | 28.4 (100) | 0.3 | 9.7 (100) |
| 7. K1060 | 18:1, 16:0 (US) | 12.8 (45) | 0.2 | 11.7 (121) |
| 8. K1060 | 18:1, β-OH-14:0 (UH) | 30.3 (107) | 0.4 | 10.5 (108) |
| 9. K1060 | 18:1, 16:0, β-OH-14:0 (USH) | 12.8 (45) | 0.2 | 11.4 (118) |
| 10. K1060[f] | 18:1, 12:0, 14:0, 16:0, β-OH-14:0 (USH) | 10.7 (38) | 0.1 | 8.3 (86) |

[a] Nanocuries per milligram was calculated by multiplying the total fatty acid radioactivity obtained from the cell per milligram of total cell protein times the relative amount of radioactivity that migrated as a given fatty acid ester class on a single thin-layer chromatograph. The relative amount of radioactivity in a fatty acid ester class was reproducible within 2% between two separate runs of the same extract. A reasonable error for the protein determination is 5%. The numbers in parentheses express the nanocuries per milligram obtained for a given fatty acid ester class in a given culture as a percentage of the value for the corresponding class measured in the control culture (culture 1 for AB1623 and culture 6 for K1060). [b] See legend to Table I. [c] These cultures were supplemented with DL-β-hydroxymyristate. Essentially identical results were obtained when the D(−) isomer was substituted for the racemic mixture as supplement. [d] 18:1 used as supplement is *cis*-Δ[9]-18:1. [e] The cultures listed in this table are the same as the respective cultures in Table I. [f] This K1060 culture was grown in a separate experiment together with a K1060 control culture supplemented with 18:1. The results were normalized to permit comparison with the other K1060 cultures shown. The 18:1-supplemented control culture (not shown) had values of 28.4, 0.5, and 7.8 nCi per mg for the SFA, UFA, and HFA classes, respectively.

ming the percentage errors in the determinations of radioactivity and of amount (micrograms).

*Fatty Acid Composition of Phosphatidylethanolamine and the Positional Distribution of Acyl Groups in this Phospholipid.* Cells of strains AB1623-1 and K19 were grown at 37° in 30-ml cultures as described above for radioisotopic labeling except that L-leucine, L-isoleucine, L-valine, and potassium acetate were absent from the medium and fatty acid supplements were each present at a concentration of 100 μg/ml. After harvesting the cells from late exponential growth phase and washing once with 0.01 M potassium phosphate (pH 7.0), the lipids were extracted by the procedure of Bligh and Dyer (1959) and chromatographed on 0.25-mm silica gel G (Anal Tech), predeveloped with acetone to activate, using chloroform–methanol–acetic acid (65:25:8). Very light exposure (<5 sec)[1] to iodine vapors was used to reveal the position of the lipids which migrate with the following $R_F$ values: 0.35, phosphatidylethanolamine; 0.59, phosphatidylglycerol; 0.94, cardiolipin; and 1.00, fatty acid. After allowing the plates to stand in room air overnight so that the bound iodine was completely released, the phosphatidylethanolamine was extracted from the silica gel by the method of Bligh and Dyer (1959). The fatty acid composition and the positional distribution of acyl groups in this phospholipid were determined as described previously (Silbert, 1970) with the following difference. At the end of the incubation of phosphatidylethanolamine with phospholipase A, the ether layer was evaporated (by placing the reaction tube in warm water) and the lipids were extracted according to the method of Bligh and Dyer (1959) except that the temperature was reduced to 0° just prior to removal of the chloroform layer (this modification assures complete recovery of lysophospholipid as well as free fatty acids in this phase). When fatty acid esters were obtained from the phosphatidylethanolamine of cells grown with trans unsaturated fatty acid supplements, the fatty acid esters were first chromatographed on 0.25-mm silica gel G (Merck Darmstadt)–12.5% silver nitrate with chloroform–0.75% ethanol to separate saturated, *trans*-monoenoic, and *cis*-monoenoic fatty acid esters. The $R_F$ values for these classes were 0.80, 0.63, and 0.40, respectively. The fractions were scraped into a conical centrifuge tube containing a standard amount of methyl pentadecanaoate. The methyl esters were eluted by extraction of the silica gel with ethyl ether, and the relative amounts of the resolved saturated, trans unsaturated, and cis unsaturated fatty acid esters were determined with respect to the pentadecanoate internal standard by gas–liquid chromatography.

*Analytical Procedures.* Amino groups released after acid hydrolysis of the cell pellets were measured on an aliquot of the hydrolysate by the ninhydrin method of Troll and Cannon (1953). Leucine was used as a standard. The micromoles of primary amino groups were converted to the equivalent milligram of protein by dividing the leucine equivalents obtained by 10.2 (Silbert *et al.*, 1972).

## Results

*Effect of Exogenous Fatty Acids on Incorporation of [$^{14}$C]-Acetate into the Different Fatty Acid Classes.* Thin-layer chromatographic fractionation of fatty acid ester classes provides a convenient method for detecting effects of fatty acid supplementation on saturated, unsaturated, and/or β-hydroxy fatty acid synthesis, as monitored by [$^{14}$C]acetate in-

[1] Selective loss of *cis*-monoenoic fatty acids occurs if the phospholipid is exposed to iodine vapors for prolonged periods (minutes) or if bound iodine is present during the preparation of fatty acid esters with 2% sulfuric acid in methanol.

TABLE IV: Effect of Fatty Acid Supplement on Fatty Acid Composition of Phosphatidylethanolamine.[a]

| Fatty Acid Composition | AB1623-1 Cultures | | | | | | | | K19 Cultures | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | None | 16:0 | 17:0 | *trans*-$\Delta^{11}$-18:1 | *cis*-$\Delta^{9}$-16:1 | *cis*-$\Delta^{10}$-17:1 | *cis*-$\Delta^{9}$-18:1 | *cis*-$\Delta^{11}$-18:1 | None | 14:0 | 15:0 | 17:0 | 18:0 | *cis*-$\Delta^{9}$-14:1 |
| Saturated fatty acids | | | | | | | | | | | | | | |
| 14:0 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 4 | 9 | 2 | 2 | 3 | 4 |
| 15:0 | | | | | | | | | 2 | 1 | 17 | | 1 | |
| 16:0 | 32 | 60 | 7 | 18 | 31 | 24 | 25 | 22 | 45 | 39 | 36 | 23 | 33 | 51 |
| 17:0 | | | 53 | | | | | | | | | 36 | | |
| 18:0 | | | | | | | | | | | | | 22 | |
| Total | 34 | 61 | 61 | 19 | 32 | 25 | 27 | 24 | 51 | 49 | 55 | 61 | 59 | 55 |
| Cis unsaturated fatty acids | | | | | | | | | | | | | | |
| 14:1 | | | | | | | | | | | | | | 9 |
| 16:1[b] | 41 | 33 | 32 | 32 | 47 | 29 | 31 | 34 | 32 | 30 | 29 | 29 | 28 | 24 |
| 17:1 | | | | | | 26 | | | | | | | | |
| 18:1 | 24 | 6 | 6 | 32 | 20 | 19 | 42 | 42 | 16 | 22 | 17 | 10 | 13 | 12 |
| Total | 65 | 39 | 38 | 64 | 67 | 74 | 73 | 76 | 48 | 52 | 46 | 39 | 41 | 45 |
| Trans unsaturated fatty acids | | | | | | | | | | | | | | |
| 18:1 | | | | 17 | | | | | | | | | | |

[a] All values are expressed as weight percentage of total fatty acids in phosphatidylethanolamine. [b] Unsaturated fatty acids and their cyclopropane derivatives are included together.

corporation. Two different *E. coli* K-12 strains are used for these studies. With AB1623, the effect of a given fatty acid supplement on the synthesis of all three types of fatty acid can be examined. On the other hand, the use of an unsaturated fatty acid biosynthetic mutant, K1060, focuses attention on the regulation of saturated and $\beta$-hydroxy fatty acid biosynthesis and avoids the problem of radioactivity from cyclopropane derivatives of unsaturated fatty acids chromatographing with saturated fatty acids. The data in Table II (and Table III) are presented as nanocuries per milligram of cellular protein. The justification for expressing the distribution of radioactivity in this way is discussed under Materials and Methods. Percentage of radioactivity in each class of fatty acid for a given culture can be calculated from this data but such numbers are interdependent and do not readily allow comparison of different cultures with respect to the extent of synthesis of a specific fatty acid or class of fatty acids.

The presence of a *cis*-monoenoic acid supplement (*cis*-$\Delta^{9}$-18:1), as previously shown for AB1623 (Silbert *et al.*, 1972), reduced extensively unsaturated and only slightly saturated and hydroxy fatty acid biosynthesis (Table II, culture 2). A saturated fatty acid supplement (16:0) reduces the incorporation of precursor acetate into saturated fatty acids by at least 46% in AB1623, the full extent being masked by cyclopropane fatty acid esters present in this fraction (see Table III, culture 3), and by 55% in K1060 (Table II, culture 7). $\beta$-Hydroxy fatty acid (DL- or D(−)-$\beta$OH14:0) has little or no effect on fatty acid synthesis in strains AB1623 Table II, cluture 4) and K1060 (Table II, culture 8). Multiple supplementation with unsaturated (*cis*-18:1), saturated (16:0 or 12:0, 14:0, 16:0), and $\beta$-OH-14:0 has the same effect on fatty acid synthesis as saturated fatty acid supplementation alone (Table II, culture 5 *vs.* 3 for AB1623 and culture 9 and 10 *vs.* 7 for K1060 cultures). Thus, it is not possible to totally stop fatty acid biosynthesis by supplementation with all the major fatty acids found in *E. coli* complex lipids or by a combination of this approach and a genetic defect in the unsaturated fatty acid pathway.

*Specificity of the Effect of Supplementation with Respect to Synthesis of Individual Fatty Acids.* The distribution of radioactivity by class found by gas–liquid chromatographic analysis (Table III) corresponds very closely to that found by thin-layer chromatography (Table II), allowing for the discrepancy caused by thin-layer chromatographic behavior of cyclopropane derivatives of unsaturated fatty acids. This agreement between the results of the two methods serves as a control on recovery and estimation of radioactivity in particular fatty acids or classes of fatty acids.

The effects of *cis*-$\Delta^{9}$-18:1 supplementation of AB1623 cultures is a reduction in synthesis not only of *cis*-$\Delta^{11}$-18:1, but also of *cis*-$\Delta^{9}$-16:1 and to a lesser extent of 16:0 and 14:0 (Table III, culture 2 and Silbert *et al.*, 1972). When 16:0 is used as exogenous supplement, there is a decrease in the synthesis of 14:0 and 16:0 of 23 and 70%, respectively, for AB1623 cultures (Table III, culture 3) and of 34 and 88%, respectively, for K1060 cultures (Table III, culture 7). $\beta$-Hydroxy and overall unsaturated fatty acid synthesis are not appreciably affected. Interpretation of the distribution of radioactivity in the unsaturated fatty acids is complicated by an unidentified component which possibly arises from modification of the cyclopropane derivatives during the isolation by acid hydrolysis (Silbert *et al.*, 1972). Thus, an effect of 16:0 on synthesis of 18:1 could be obscured here but is clearly evident below (Table IV) in the fatty acid composition of phospho-

TABLE V: Positional Distribution of Fatty Acids in Phosphatidylethanolamine.[a]

| Fatty Acid Composition | AB1623-1 Cultures | | | | | | | | K-19 Cultures | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | None | 16:0 | 17:0 | *trans*-$\Delta^{11}$-18:1 | *cis*-$\Delta^{9}$-16:1 | *cis*-$\Delta^{10}$-17:1 | *cis*-$\Delta^{9}$-18:1 | *cis*-$\Delta^{11}$-18:1 | None | 14:0 | 15:0 | 17:0 | 18:0 | *cis*-$\Delta^{9}$-14:1 |
| Position 1 | | | | | | | | | | | | | | |
| 14:0 | 3 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 4 | 10 | 12 | 1 | 2 | 5 |
| 15:0 | | | | | | | | | 3 | 2 | 31 | 1 | 1 | 2 |
| 16:0 | 65 | 96 | 13 | 34 | 59 | 49 | 50 | 43 | 86 | 77 | 54 | 38 | 57 | 89 |
| 17:0 | | | 85 | | | | | | | | | 60 | | |
| 18:0 | 2 | | | 1 | 1 | | 1 | | | | | | 37 | 1 |
| Total SFA[b] | 70 | 97 | 99 | 37 | 62 | 51 | 53 | 45 | 93 | 89 | 97 | 100 | 97 | 97 |
| 14:1 | | | | | | | | | | | | | | |
| 16:1[c] | 8 | 1 | | 6 | 15 | 4 | 7 | 7 | 1 | 1 | | | | |
| 17:1 | | | | | | 27 | | | | | | | | |
| 18:1 | 22 | 2 | 2 | 32 | 23 | 17 | 39 | 48 | 5 | 9 | 2 | | 1 | 3 |
| Total *cis*-UFA | 30 | 3 | 2 | 38 | 38 | 48 | 46 | 55 | 6 | 10 | 2 | 0 | 1 | 3 |
| *trans*-18:1 | | | | 25 | | | | | | | | | | |
| Position 2 | | | | | | | | | | | | | | |
| 14:0 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 3 | 9 | 1 | 3 | 2 | 3 |
| 15:0 | | | | | | | | | | | 10 | | | |
| 16:0 | 4 | 30 | 3 | 2 | 2 | 2 | 3 | 2 | 10 | 5 | 6 | 7 | 9 | 11 |
| 17:0 | | | 23 | | | | | | | | | 10 | | |
| 18:0 | | | | | | | | | | | | | 6 | |
| Total SFA | 6 | 31 | 28 | 3 | 3 | 3 | 4 | 3 | 13 | 14 | 17 | 20 | 17 | 14 |
| 14:1 | | | | | | | | | | | | | | 12 |
| 16:1 | 71 | 61 | 65 | 56 | 79 | 52 | 53 | 60 | 65 | 62 | 56 | 71 | 62 | 59 |
| 17:1 | | | | | | 24 | | | | | | | | |
| 18:1 | 23 | 8 | 6 | 32 | 18 | 21 | 44 | 37 | 22 | 24 | 26 | 10 | 21 | 16 |
| Total *cis*-UFA | 94 | 69 | 71 | 88 | 97 | 97 | 97 | 97 | 87 | 86 | 82 | 81 | 83 | 87 |
| *trans*-18:1 | | | | 9 | | | | | | | | | | |

[a] All values are expressed as weight percentage of total fatty acids in each position. Position 1 represents the fatty acids remaining on the lysophosphatidylethanolamine after treatment with phospholipase A as described in Methods; position 2, the fatty acids released by the enzyme. For all cultures examined, the composition *calculated* for the total phosphatidylethanolamine from that measured here for the individual positions agreed very closely with that *determined directly* from the phosphatidylethanolamine not exposed to the enzyme (see Table IV). [b] See legend to Table I. [c] See footnote *b* in legend to Table IV.

lipid from AB1623-1 cultures supplemented with 16:0 (or 17:0) and K19 cultures supplemented with 17:0 (or 18:0).

As noted above in the thin-layer fractionation, hydroxy fatty acid alone or together with unsaturated and/or saturated fatty acids has little effect on its own synthesis (Table III, K1060 cultures 8 and 9). Furthermore, since the specific radioactivities of $\beta$-hydroxymyristate in cultures *supplemented* with this fatty acid is not decreased significantly (that is, diluted by unlabeled exogenous acyl chains) relative to the corresponding values obtained from the control cultures, there can be little incorporation of this supplement into complex lipids (Table I, cultures 8 and 9 *vs.* 6).

*Replacement of Endogenously Derived Fatty Acids in Phospholipid by Analogs Provided as Supplements.* AB1623-1 and K19, related to AB1623 and K1060, respectively, are both defective in long-chain fatty acid degradation but normal with respect to fatty acid synthesis. Exogenous fatty acids are incorporated into phospholipid in these two strains without alteration (see also Silbert *et al.*, 1968). If fatty acids that are not normally synthesized and that are easily recognized by chromatographic methods are used as supplements, for example, fatty acids with an odd number of carbons or with a *trans*-ethylenic bond, then the relative amounts of fatty acid derived from synthesis and the exogenous source can be readily distinguished. Table IV gives the fatty acid composition of phosphatidylethanolamine isolated from AB1623-1 and K19 cultures grown without fatty acid supplement or in the presence of a saturated, trans unsaturated or cis unsaturated fatty acid. A conspicuous difference between the two strains is the relative deficiency in saturated fatty acids in the phospholipid derived from AB1623-1 when comparing unsupplemented control cultures of AB1623-1 and K19 grown at 37°. On supplementation of the growth medium with long-chain saturated fatty acids, AB1623-1 utilizes the supplement more extensively and the difference in fatty acid composition between the two strains disappears. Considering together the results obtained in cultures of both strains, the fatty acid composition of phosphatidylethanolamine is enriched with respect to the fatty acid provided as exogenous supplement. In the case of fatty acids of unusual structure (15:0, 17:0, 18:0, *trans*-18:1, *cis*-17:1) which can be derived only from the exogenous source, the full extent of utilization can be observed

TABLE VI: Distribution of Specific Fatty Acids between Positions 1 and 2 of Phosphatidylethanolamine (expressed as % in position 1).[a]

| Specific Fatty Acid | AB1632 Cultures | | K19 Cultures | |
|---|---|---|---|---|
| | Fatty Acid Arising Solely from Synthesis | Fatty Acid Arising from Synthesis and/or Exogenous Source | Fatty Acid Arising Solely from Synthesis | Fatty Acid Arising from Synthesis and/or Exogenous Source |
| 14:0 | 67 | | 55 | 55 |
| 15:0 | | | 90 | 76 |
| 16:0 | 94 | 76 | 91 | |
| 17:0 | | 78 | | 86 |
| 18:0 | (100)[b] | | (100) | 87 |
| *cis*-$\Delta^9$-14:1 | | | | 0 |
| *cis*-$\Delta^9$-16:1 | 10 | 34 | 2 | |
| *cis*-$\Delta^{10}$-17:1 | | 53 | | |
| *cis*-$\Delta^9$-18:1 | | 47 | | |
| *cis*-$\Delta^{11}$-18:1 | 49 | 56 | 18 | |
| *trans*-$\Delta^{11}$-18:1 | | 74 | | |

[a] Data presented in Table V recalculated to show for specific fatty acids the per cent in position 1 relative to the total amount of the particular fatty acid present in the phospholipid from unsupplemented cultures or cultures supplemented with that fatty acid. [b] Numbers in parentheses are values calculated from weight percentages <1% and where all the fatty acid is found in position 1.

directly. Utilization of long-chain saturated fatty acids (15:0, 17:0, 18:0) and *trans*-18:1 is associated with the replacement of 16:0. In the case of 16:0 and 17:0 supplemented cultures, the effect is pleotropic in that the phospholipid content of 18:1 and to a lesser extent of 16:1 is reduced. *trans*-18:1 utilization (AB1623-1 culture) results in a compensating increase and decrease in *cis*-18:1 and *cis*-16:1, respectively. Only modest changes in overall fatty acid composition of phospholipid occurs in response to supplementation with *cis*-16:1 (AB1623-1 culture) or with *cis*-14:1 (K19 culture). Incorporation of longer-chain cis unsaturated fatty acids (17:1 and 18:1) is associated with reductions in the phospholipid content of 16:0 as well as 16:1.

The overall changes in fatty acid composition discussed above are the consequence of the positional distribution of endogenously derived fatty acids manifest in the unsupplemented cultures and how this pattern is affected by the incorporation of exogenous fatty acid into one or both positions of the phospholipid. These data are shown in Tables V and VI. Exogenous long-chain fatty acids, including saturated, trans unsaturated, and cis unsaturated fatty acids containing more than 14 carbons, decrease the phospholipid content of 16:0 derived from synthesis by replacing this fatty acid in position 1. The reduction in the phospholipid content of cis unsaturated fatty acids produced by the cell (16:1 and 18:1) results from incorporation of the fatty acid supplement into position 1 and/or position 2.

Utilization of 17:0 mimics that of 16:0 in extent of incorporation, positional distribution, and overall effect on the incorporation into phospholipid of fatty acids derived from synthesis (see Tables V and VI). Thus, it is a suitable nonradioactive label for studying how exogenous 16:0 affects its own synthesis. AB1623-1 and K19 cultures supplemented with 17:0 show a fourfold (32–7%) and a twofold (45–23%) reduction in the content of 16:0 found in phosphatidylethanolamine when compared to that present in the phospholipid isolated from unsupplemented control cultures. Exogenous 17:1 is incorporated into phospholipid with the same positional distribution as 18:1 (see Table VI). However, its incorporation results in greater replacement of 16:1 than 18:1 (see Tables IV and V). Thus, it is not a perfect analog of either 16:1 or 18:1 in its incorporation behavior, and it cannot be used to determine what effect exogenous 16:1 or 18:1 has on its own synthesis.

## Discussion

In a previous study (Silbert *et al.*, 1972) it was concluded that the reduced radioactivity derived from [$^{14}$C]acetate and recovered in certain fatty acids in response to exogenous unsaturated fatty acid supplementation of AB1623 and K1060R (a revertant of K1060 to wild-type fatty acid synthesis) was, in fact, due to decreased synthesis and not to degradation or excretion. The present experiments examine the effect of saturated fatty acid supplementation on fatty acid biosynthesis. Our results are in agreement with the conclusions of Sinensky (1971) who demonstrated extensive utilization of exogenous 16:0, based on a decrease in the specific radioactivity of 16:0 relative to 18:1 in the phospholipid of cells exposed to uniformly labeled [$^{14}$C]glucose or [$^3$H]acetate during exponential growth. In the present study we used the incorporation of [$^{14}$C]acetate into specific fatty acids per unit weight of cellular protein as a measure of synthesis. This approach has the following advantages. It becomes possible to examine directly the effect of supplementation not only on the synthesis of the fatty acid corresponding to the one provided in the medium but also on the endogenous production of other fatty acids which might be effected simultaneously. Specific radioactivity measurements alone are hard to interpret in terms of synthesis. For a fatty acid which can be derived from the supplement, a decreased specific radioactivity is compatible with incorporation of the exogenous unlabeled fatty acid with or without a decrease in synthesis. For a fatty acid which cannot be derived from the exogenous supplement, a reduced rate of synthesis would be reflected in a decreased specific radioactivity only early in the labeling period and would become less apparent as the pulse period extended over many generations. One disadvantage in the approach taken in the present study is that errors inherent in the experimental procedure make small differences (<20%) in incorporation of radioactivity probably of little significance.

Although the greatest effects of supplementation are usually seen in the synthesis of the fatty acid corresponding to that provided in the medium, the endogenous production of other fatty acids is also reduced. The effect of the exogenous fatty acid on synthesis may be a direct one or secondary to the accumulation of endogenously derived acyl thioesters following the preferential incorporation of the exogenous supplement. The latter hypothesis would be the simplest since it would apply also to regulation in unsupplemented cultures. Thus, we envision that the acyl transferases involved in glycerophosphatide synthesis represent a primary control site

which determine not only how the phospholipid is structured with fatty acid (Sinensky, 1971) but subsequently what fatty acids are synthesized. To the extent that an exogenous fatty acid is recognized by these enzymes and placed into one or both positions of the phospholipid, those endogenously derived fatty acids normally put into these sites are kept out and in unknown fashion must shut down their own synthesis.

In the course of these studies we have observed that there is more unsupplemented AB1623-1 cultures grown at 37° than cis unsaturated fatty acids in the phospholipid derived from in the phospholipid from other *E. coli* K-12 strains (see also Silbert, 1970) grown under similar conditions. This difference is associated with a greater incorporation of *cis*-monoenoic fatty acids into position 1. These findings suggest that there may be strain differences at 37° in the specificity of the acyl transferase(s) participating in phospholipid biosynthesis or in the relative rates of saturated *vs.* unsaturated fatty acid synthesis.

Although extensive replacement of those fatty acids present in phospholipid (especially 16:0, 16:1, and 18:1) can be achieved by exogenous supplementation, the same is not true for those acyl groups found in lipid A (12:0, 14:0, and βOH-14:0). The presence of β-hydroxymyristate in the cultures has not been effective in reducing synthesis of that fatty acid although the cell can grow on it as carbon source (Dr. Robert Bell, personal communication) and, therefore, can transport and activate this fatty acid. The failure to incorporate exogenous acyl groups into lipid A but not into phospholipid suggests that the acyl transferase activity involved in synthesis of the former complex lipid may show a greater specificity for the acyl-ACP which can be derived possibly only from *de novo* synthesis.

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# Role of Sulfhydryl Groups in Erythrocyte Membrane Structure†

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ABSTRACT: The sulfhydryl binding reagent *p*-chloromercuribenzenesulfonate extracts up to 40% of the protein present in hypotonically lysed, extensively washed red cell "ghost" membranes. The process is rapid, shows no temperature dependence in the range of 15–37°, and is not reversed by dithiothreitol added after extraction. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate demonstrates that all major peptides are extracted in part except for the predominant species which remains entirely in the membrane. Only one minor glycoprotein is solubilized. Coincident with the elution of protein, there is breakdown of the "ghosts" into smaller vesicles. The extracted membranes are more soluble than untreated "ghosts" in Triton X-100 and aggregate spontaneously in the presence of 0.1–0.4 M NaCl. *N*-Ethylmaleimide-treated membranes show the same physical changes although there is no significant release of protein. It is suggested that sulfhydryl groups play a role in hydrophobic bonding of proteins to membranes and that many of the effects attributed to sulfhydryl binding agents may be related to nonspecific structural alterations in the membrane.

Many functions associated with cellular membranes have been shown to be inhibited by reagents that bind free sulfhydryl groups (Garrahan and Rega, 1967; Makinose, 1969; Scott *et al.*, 1970; Sutherland *et al.*, 1967; van Steveninck *et al.*, 1965; Janacek, 1962; Carter and Martin, 1969a; Rothstein, 1970). Generally, this has been interpreted as indicating that a given class of sulfhydryl groups is intimately involved in the process under study, either a part of the critical membrane protein (enzyme, transport protein, antigen, etc.) or closely associated with it in the membrane. In at least one case, that involving the β-galactoside "permease" of *Escherichia coli*, this specificity has been confirmed by the radioactive

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