Difference Decoupling Nuclear Magnetic Resonance: A Method to Study the Exchange of Fatty Acids between Phospholipid Molecules†

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ABSTRACT: A nuclear magnetic resonance technique has been developed to study the exchange of oleic acid moieties between phospholipid molecules of *Escherichia coli*. The method relies on the splitting of the NMR spectrum of the proton on position 2 of a diglyceride glycerol backbone caused by a ^{13}C atom in the carboxyl of the fatty acid esterified to position 2. By subtracting decoupled from coupled spectra, the amount of diglycerides having ^{13}C in the carboxyl of the position 2 fatty acid moiety can be measured. An *E. coli* strain designed to optimize incorporation of exogenous glycerol and oleate is grown on [2-^{2}H]glycerol and [1-^{13}C]oleate (phase 1). Diglycerides prepared from the phospholipids of the cells have no position 2 proton spectrum due to the deuterium substitution. The cells are then switched to a medium having unenriched glycerol and oleate supplements and allowed to continue growth (phase 2). Diglycerides from phospholipids synthesized de novo during growth in the second medium will have no ^{13}C splitting. Therefore if a proton resonance with ^{13}C splitting is observed, then this splitting must result from transfer of a [1-^{13}C]oleate moiety from a phospholipid synthesized in phase 1 to a phospholipid molecule synthesized in phase 2 (no significant intracellular pools of either glycerol or oleate are found in these cultures). We have used these techniques to measure the exchange of oleic acid moieties between phospholipid molecules in both normally growing cultures of *E. coli* and cultures starved for oleic acid. In neither case did we observe fatty acid exchange. The implications of this result and the general usefulness of the technique are discussed.

There are a number of situations in the study of biosynthetic mechanisms in which a method to measure not only the incorporation of the component atoms of a precursor but also to monitor the integrity of preexisting or of newly formed bonds between these atoms is needed. Particularly challenging problems arise when reactant and product are chemically very similar or identical. Such reactions can be characterized as exchange reactions.

A possible example of such an exchange reaction is the exchange of fatty acids among phospholipid molecules thought to occur during the growth of *Escherichia coli*. This postulate of fatty acid exchange among preexisting and newly synthesized lipids stems from rather indirect experimental evidence collected in a number of laboratories (for examples, see Okuyama, 1969; Aibara et al., 1972; Scandella and Kornberg, 1971; Nunn and Cronan, 1974). Such exchange has been thought to occur by a deacylation–reacylation sequence similar to that proposed by Hill and Lands (1970) in mammalian systems, although, since phospholipid fatty acid moieties are metabolically stable in *E. coli* (Cronan and Vagelos, 1972), the deacylation and reacylation reactions would have to be rather more tightly coupled than in the mammalian systems.

If fatty acid exchange does indeed occur between phospholipid molecules in *E. coli*, then the interpretation of various experiments using fatty acid auxotrophs of this organism would become much less straightforward. These auxotrophs have been a very useful system to test various questions of membrane genesis, structure, and function (for review, see Cronan and Gelmann, 1975). It has generally been assumed in these investigations that the phospholipid fatty acid composition of *E. coli* is determined solely by phospholipid synthesis rather than by a combination of synthesis and fatty acyl moiety exchange. However, this assumption has not been rigorously tested since the exchange of fatty acids between intact phospholipid molecules is particularly difficult to measure. Radioactive labeling procedures are ineffective unless a method to separate preexisting phospholipid molecules from newly synthesized molecules is available. Isopycnic centrifugation techniques, coupled with density labels, are generally used for such experiments, but the low molecular weight and micellar properties of phospholipid molecules limit application of such techniques to phospholipids. We have therefore approached this problem using nuclear magnetic resonance (NMR)[1] techniques. In this paper, we present an NMR method to assay such exchange reactions and the application of the assay method to the question of fatty acid exchange between *E. coli* phospholipid molecules.

Materials and Methods

Bacterial Strain. Strain CY169, *E. coli* K12 strain, was derived by the following sequence of genetic crosses. K1060, a *mel*$^{-}$, *fabB, fadE* strain (Overath et al., 1970, 1971), was mated with strain X340 (HfrC, *proB, metB*) and *mel*$^{+}$ recombinants were selected. CY168, one of the *metB, fabB, fadE* recombinants, was mated with strain BB20-14, an HfrC, *gpsA, glpD* strain (Bell, 1973; Cronan and Bell, 1974), and *metB*$^{+}$ recombinants were selected. The final strain, CY169, has the following genotype, *fabB5, fabE62, gpsA20, glpD8*. This strain is unable to synthesize or degrade *sn*-glycerol 3-phosphate due to the *gpsA* and *glpD* mutations. Strain CY169 thus has an absolute growth requirement for both an appropriate unsaturated fatty acid (such as oleate) and either glycerol or *sn*-glycerol 3-phosphate.

Bacterial Growth and Lipid Extraction. Strain CY169 was grown in a standard medium of medium E (Vogel and Bonner,

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[1] Abbreviation used: NMR, nuclear magnetic resonance.

1956) supplemented with: sodium succinate, 0.5%; acid-hydrolyzed casein, 0.05%; glycerol, 0.02%; potassium oleate, 40 mg/L; and Brij 58 detergent, 0.05%. When indicated, at glycerol and oleate were replaced by [2-^{2}H]glycerol and potassium [1-^{13}C]oleate at the same concentrations. The generation time of CY169 in this growth medium was about 100 min with either enriched or nonenriched supplements. Cultures were grown at 37 °C with aeration in a gyrorotary shaker. Cultures to be transferred from one growth medium to another were centrifuged, resuspended in medium E plus 0.05% Brij, and recentrifuged. The cells were then suspended in the new medium and grown further at 37 °C.

For lipid extraction, the cells were collected by centrifugation, washed with distilled water, resuspended in distilled water, and extracted with chloroform–methanol as previously described (Cronan and Wulff, 1969).

Lipid Analysis. In order to simplify the NMR spectra, the phospholipids obtained by chloroform–methanol extraction were converted to 1,2-diglycerides by hydrolysis with phospholipase C from *Bacillus cereus* as previously described (Nunn and Cronan, 1974). The phospholipase C preparation was the ammonium sulfate precipitated preparation of Zwaal et al. (1971). The conversion of phospholipids to diglycerides was monitored by thin-layer chromatography (a tracer amount of ^{14}C-labeled phospholipid was added to facilitate the quantitation of hydrolysis) and was >95%. Following hydrolysis, the diglycerides were chromatographed on a 1-g column of silicic acid and were eluted with 20 mL of chloroform. Thin-layer chromatography showed this fraction to be pure 1,2-diglycerides.

For gas chromatographic analysis, the fatty acid moieties of the diglycerides were transesterified to their methyl esters with 0.5 M sodium methoxide in methanol. The methyl esters were analyzed on a diethylene glycol succinate column at 190 °C as previously described (Gelmann and Cronan, 1972).

Isotopically Enriched Chemicals. [1-^{13}C]Oleic acid (90–91% enriched at the carboxyl carbon) was purchased from Merck Isotopes. D_2O and sodium borodeuteride were also from Merck. Dihydroxyacetone was from Sigma Chemical Co.

[2-^{2}H]Glycerol was synthesized by reduction of dihydroxyacetone with sodium borodeuteride in 99.7% deuterium oxide. Following reduction, the solution was passed through a mixed bed ion exchange resin and flash evaporated. The resulting [2-^{2}H]glycerol was fully active in the glycerol kinase and (after phosphorylation) *sn*-glycerol 3-phosphate dehydrogenase assays. NMR analysis showed the predicted 20% reduction in proton content.

[1-^{14}C]Oleic acid and [2-^{3}H]glycerol were purchased from New England Nuclear.

NMR Analysis. Except where noted, NMR samples were prepared to be approximately 0.03 M in 0.4 mL of 99.8% $CDCl_3$. Each sample was subjected to two freeze–pump–thaw cycles and sealed under nitrogen.

Spectra were obtained on a Bruker HX270 high field spectrometer in the pulse Fourier transform mode. A carbon-13 decoupling frequency originating from a General Radio 1061 frequency synthesizer was fed through a console tripler to produce a decoupling field of about 100 mG. The decoupling field was supplied continuously but shifted on an off-resonance under software control. Sets of decoupled and nondecoupled spectra were accumulated alternately using eight 90° pulses per block at a repetition rate of 4.6 s. Accumulation continued for approximately 5 h. The 8K data sets were processed with identical parameters to yield spectra of 1000 Hz width. Decoupled and nondecoupled spectra were subtracted without further adjustment to produce difference spectra. The zero and

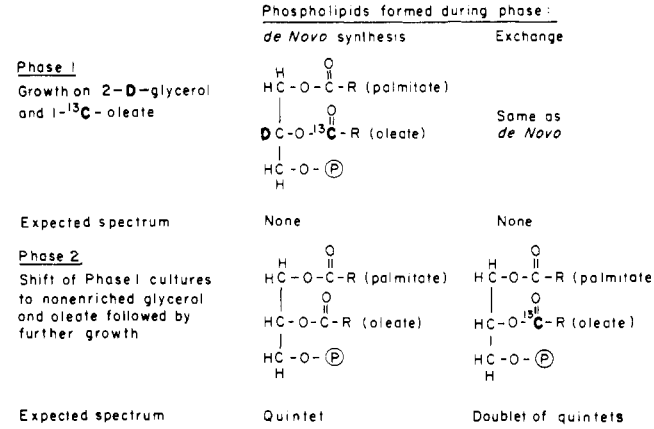

FIGURE 1: Outline of the experimental method. Cultures of strain CY169 were grown exponentially in the standard medium supplemented with [1-^{13}C]oleate and [2-^{2}H]glycerol (2-D-glycerol) for 8–12 generations (phase 1). These cultures were then centrifuged and the cell pellet was washed (as described in Materials and Methods). Diglycerides prepared from the phospholipids of these cells show no spectrum for the proton on position 2 of the glycerol moiety since the proton has been substituted with deuterium. In phase 2, the cell pellet was resuspended in the standard medium supplemented with nonenriched oleate and glycerol and allowed to resume exponential growth. Following various periods of exponential growth, samples of the cultures were harvested and diglycerides were prepared from the cellular phospholipids. In the absence of fatty acid exchange, only the quintet of the 2-position proton would be observed. In the presence of fatty acid exchange, a pair of quintets, due to splitting of the proton resonance by the carboxyl ^{13}C of the oleate exchanged into position 2, would be superimposed.

0.3 generation control spectra were fit with a computer routine summing Lorenzian lines to quantitate the ^{1}H–^{13}C lipid background level. All other spectra are scaled to these spectra using the amplitude of the unsaturated olefinic proton peak and the fatty acid analysis as determined by gas chromatography.

Results and Discussion

Description and Rationale of the NMR Method. Bond integrity can be monitored via through-bond spin–spin coupling of pairs of magnetic isotopes incorporated to preexisting molecules. The observation of ^{13}C–^{13}C coupling in microbial products has, for example, been extensively used to indicate the intact incorporation of a two carbon unit from 1,2-^{13}C-enriched acetate (McInnes et al., 1976). The appearance of enriched (but single) lines indicates bond breakage with incorporation of single atoms.

In lipid molecules breakage of the glycerol–fatty acid ester bond upon fatty acid exchange might be monitored in a completely analogous fashion using carbonyl-glycerol ^{13}C–^{13}C coupling. In principle, however, any pair of magnetic nuclei can be used in such an experiment, for example, ^{1}H–^{13}C.

There are substantial advantages in using a proton as one member of the pair. First, synthesis of deuterated substitutes for the protonated molecule is often easier than synthesis of a carbon enriched species. Second, if the effects of spin–spin coupling can be observed, then in theory an improvement of about 20-fold in sensitivity (over direct ^{13}C observation) is expected. The third advantage of proton observation is that quantitative data can be obtained without the concern of large differential Overhauser relaxation effects.

We therefore present here a detection scheme based on observation of ^{13}C satellites in the proton spectrum of a molecule produced by synthesis from a newly supplied protonated glycerol and a ^{13}C fatty acid previously incorporated into lipids having only deuterated glycerol moieties. The scheme is presented in Figure 1. Molecules synthesized in phase 1 of the

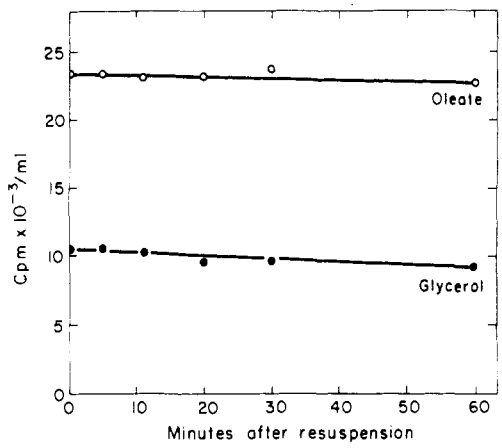

FIGURE 2: Equilibration of oleate and glycerol pools in strain CY169. Two cultures of strain CY169 were grown with 40 mg/mL potassium oleate and 0.02% glycerol described in Materials and Methods except one culture contained 2.5 μCi/mL of [1-^{14}C[oleic acid and the other culture contained 2.5 μCi/mL of [2-^{3}H]glycerol. After 20 min of exponential growth in the presence of the radioisotopes, both cultures were centrifuged and washed as described in Materials and Methods. The cells from both cultures were then resuspended in the standard medium containing nonradioactive oleate and glycerol and allowed to resume exponential growth. At various times after resuspension, 0.8-mL samples were taken into 3 mL of methanol-chloroform (2:1, v/v) and extracted as described in Materials and Methods except the chloroform phase was washed three times with 0.1 M sodium bicarbonate in 50% aqueous methanol and twice with water. The extraction with base removed all detectable free fatty acid from the phospholipid sample as deterimined by thin-layer chromatography. An aliquot of the chloroform phase was then evaporated and counted in Aquasol scintillation solution (New England Nuclear).

growth cycle will exhibit no proton resonance in the region of the spectrum corresponding to the glycerol 2-position. Molecules synthesized in phase 2 from newly supplied [^{12}C]fatty acid and [^{1}H]glycerol will exhibit resonances without ^{13}C splitting. Only those molecules which have been synthesized from new [^{1}H]glycerol and exchanged [^{13}C]fatty acid will give rise to split resonances of ^{13}C satellites. By comparing satellites and central resonance intensities, a percentage of exchange can be determined.

In practice, observation of satellites in the presence of strong central lines is difficult. Recently there have been several applications of difference techniques to the detection of satellites (Scott et al., 1976; Llinás et al., 1976). ^{1}H-^{13}C coupling may be artificially removed by strong irradiation at an appropriate ^{13}C frequency. The ^{1}H-^{13}C multiplet collapses to a single line or proton coupled multiplet. The procedure has minimal effects on position and intensity of resonances arising from sites with no adjacent ^{13}C nuclei. Therefore, taking the difference between ^{13}C-coupled and ^{13}C-decoupled proton spectra yields a spectrum having only satellite connected resonances present. We will show that the application of this technique provides a viable means of quantitating fatty acid exchange in lipid biosynthesis.

Biological System. Strain CY169, a quadruple mutant of *E. coli* K12, was used in these experiments for the following reasons. First, the formation of unsaturated fatty acids with ^{12}C in the carboxyl carbon (either by de novo synthesis or by β oxidation of the added fatty acid) would greatly increase the background of the experiments. The formation of carboxyl ^{12}C-unsaturated fatty acids by synthesis or β oxidation was prevented by the *fabB* and *fadE* blocks, respectively. For similar reasons, the synthesis of *sn*-glycerol 3-phosphate was blocked by the *gpsA* mutation. The *glpD* mutation prevents the degradation of the [2-^{2}H]glycerol. The fatty acid and the *glpD* mutations are virtually absolute. However, the *gpsA* mutation is slightly leaky (Bell, 1973; Cronan, et al., 1975) and allows about 5% of the normal rate of *sn*-glycerol 3-phosphate synthesis and, thus, introduces a background of about 5% into these experiments.

The validity of our experimental approach is dependent on an abrupt shift from the synthesis of phospholipids containing [2-^{2}H]glycerol and [1-^{13}C]oleate to phospholipid molecules containing nonenriched precursors. Thus, it is necessary that intracellular pools of both oleate and glycerol be quite small. Data in the literature indicate the pools of both precursors are small (Bell, 1973; Cronan et al., 1975; Nunn and Cronan, 1974; Overath et al., 1970); however, we felt it necessary to measure the pools of oleate and glycerol in our strain under our experimental conditions.

Cultures of strain CY169 were labeled with either [1-^{14}C]oleate or [2-^{3}H]glycerol for about 0.2 generation. The cells were then collected and washed as described in Materials and Methods and resuspended in nonradioactive medium. As shown in Figure 2, the accumulation of radioactive label is immediately halted by the washing and resuspension procedure and thus the intracellular pools of oleate and glycerol are small. From these data, the pool of either precursor contains only enough precursor for <0.01 generation of phospholipid synthesis. The shift from enriched to nonenriched precursors is therefore essentially immediate (<2 min) upon suspension of the cells in the second medium. The data in Figure 2 also show the metabolic stability of the fatty acid and glycerol moieties of the membrane phospholipids. The stability of the [^{14}C]-oleate label is in accord with earlier experiments (Cronan and Vagelos, 1972) and indicates that any existing deacylation and reacylation reactions must be rightly coupled to one another. The slight but significant turnover of the glycerol label seen in Figure 2 can be attributed to the slow turnover of the nonacylated glycerol moiety of phosphatidylglycerol which was demonstrated in *E. coli* by Kanfer and Kennedy (1964).

It should be noted that oleic acid is found almost exclusively in position 2 of the phospholipids of unsaturated fatty acid auxotrophs grown on oleic acid (Silbert, 1970). Since the oleic acid contents of all our cultures are 53–54% of the total phospholipid fatty acid (as determined by gas chromatography), virtually all the phospholipid molecules synthesized must have oleic acid in position 2. It should also be noted that the lipid composition of strain CY169 is indistinguishable from the composition reported for its parental strains K1060 (Overath et al., 1971) and BB20-14 (Bell, 1973). Only these lipid species normally found in *E. coli* were detected by thin-layer or gas chromatography.

NMR Spectra. The proton on the 2 position of a diglyceride glycerol backbone normally gives rise to a multiplet structure centered 5.0 ppm downfield from Me_4Si. That multiplet is approximately a quintet due to a 5.0 Hz coupling with the four glycerol backbone protons. When a carbon-13 atom is present at the carboxyl carbon esterified to the 2 position of the backbone an additional 3.2 Hz coupling results. Figure 3a shows the resulting multiplet in diglyceride about 80% enriched with ^{13}C in the fatty acid carboxyl groups. ^{13}C decoupling reduces this structure to the normal quintet as illustrated in Figure 3b. The difference between the two spectra is displayed in Figure 3c.

In a sample having low levels of ^{13}C incorporation (10%) such as that in Figure 4, the effects of the additional ^{13}C coupling are not visually apparent because of the dominance of the quintet arising from the ^{12}C-containing diglycerides. However, taking the difference between decoupled and nondecoupled spectra efficiently removes these unperturbed signals, and the amplitude of the residual difference spectrum can

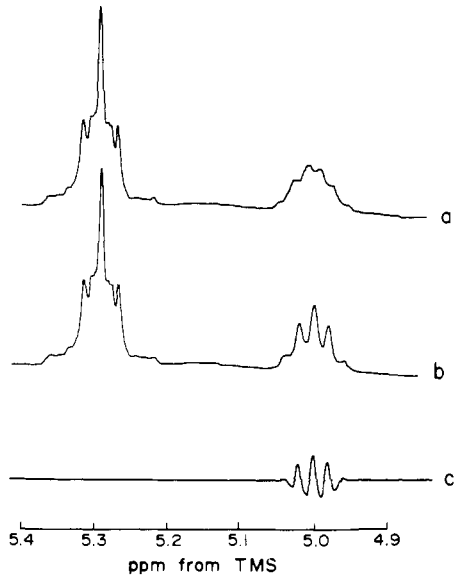

FIGURE 3: NMR spectra of *E. coli* derived diglycerides. The diglycerides are approximately 80% enriched at the carboxyl esterified to the 2 position of the glycerol moiety. (a) Spectrum without ^{13}C decoupling; (b) spectrum with ^{13}C decoupling; (c) difference spectrum (b-a). The multiplet at 5.2 ppm in spectra b and c arises from the olefinic protons of the unsaturated fatty acyl moieties of the diglycerides.

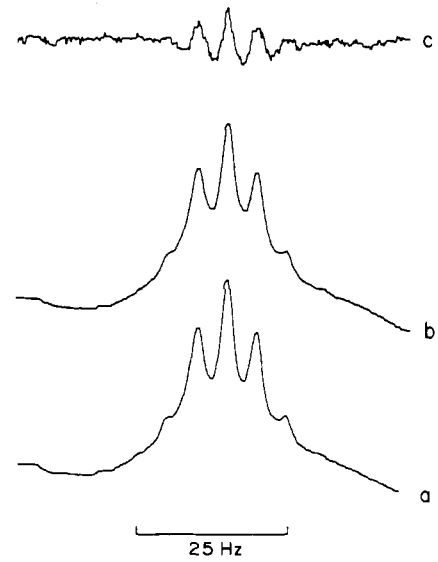

FIGURE 4: Diglyceride glycerol-H_2 multiplet. The sample is a $CDCl_3$ solution 0.07 M in diglyceride 10% enriched at the carboxyl esterified to the 2 position. (a) Spectrum with ^{13}C decoupling; (b) spectrum without ^{13}C decoupling; (c) difference spectrum amplified by a factor of 4.

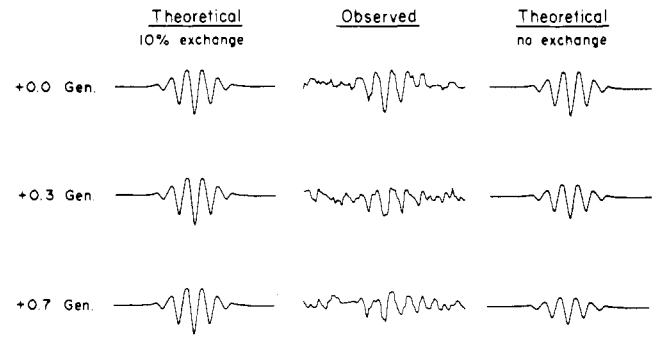

FIGURE 5: Theoretical and observed difference spectra for diglyceride samples. Theoretical spectra are shown for (left) 10% exchange and (right) for no exchange. See text for further explanation. The experimental spectra are of diglyceride samples derived from *E. coli* cultures harvested after 0.0, 0.3, or 0.7 generations of growth in phase 2.

be directly related to the percentage of ^{13}C incorporation. Quantitation of ^{13}C incorporation requires computer simulation of the spectra because of partial overlap of the decoupled and nondecoupled resonance line positions. In spectra of *E. coli* products such as those in Figure 3, the efficiency of the difference technique can be evaluated by the complete disappearance of the multiplet at 5.3 ppm which arises from olefinic protons on the unsaturated fatty acid moieties of the diglycerides.

Difference spectra for diglyceride samples prepared from *E. coli* lipids grown on [^2H]glycerol and [^{13}C]oleic acid during the first phase of growth and on [^1H]glycerol and [^{12}C]oleic acid in the second phase (terminated at 0, 0.3, and 0.7 additional generation) are presented in Figure 5. All spectra have been scaled using the olefinic multiplet and gas-chromatograph-determined fatty acid compositions as a standard. Scaling factors agree with predictions based on variation in sample concentration and accumulation time. Of the three samples only the 0.3 and 0.7 generation samples are capable of showing fatty acid exchange. The zero-time sample is a necessary measurement of background for the experiment. Computerized fits of the difference spectra show that about 6% of all diglycerides have a protonated glycerol-[^{13}C]fatty acid pair. That this level is different from zero can be attributed to the leakiness of the *gpsA* mutation (see above) and to traces of 2-position protons in the [2-^2H]glycerol.

Separation of cells from the phase 1 growth medium as described in the Materials and Methods section has been shown to leave no detectable intracellular pool of either fatty acid or glycerol (Figure 2). Changes in the amplitude of the difference spectra upon further growth in the presence of [^{12}C]fatty acid and [^1H]glycerol can therefore be interpreted on the basis of fatty acid exchange. We will consider two possible results. First, if no exchange of fatty acid occurs, then the synthesis of ^1H-^{12}C lipid will (when scaled to total lipid) *decrease* the difference spectra by ~20% and 40% after growth for 0.3 and 0.7 generation, respectively. Second, if >10% of the oleate moieties in position 2 of the phospholipids formed in phase 2 are derived from previously existing (^2H-^{13}C) phospholipid during a generation of growth, then the difference spectra should *increase* by >6% and >5% after 0.3 and 0.7 generation, respectively.

As shown in Figure 5, the experimental difference spectra decrease and the magnitude of this decrease is that calculated if no (or <10%) exchange of oleate moieties occurs between phospholipid molecules. It should be noted that duplicate spectra on a given sample were identical in all cases examined. Four independent experiments were done. The first experiment indicated a small (~10%) amount of exchange. However, the subsequent three experiments gave results identical with those presented. The apparent result of the first experiment is attributed to inefficient removal of [^{13}C]oleate from the cell pellets which in turn was due to the large sample size needed to obtain spectra on the 90-MHz instrument then used. We therefore conclude that there is no evidence of fatty acid exchange when unsaturated fatty acid is available in sufficient levels. This result is in accord with that of Overath et al. (1971) who used a less direct technique.

Exchange during Oleate Starvation? From radioactive labeling experiments, Nunn and Cronan (1974) suggested that unsaturated exchange might occur during starvation of an unsaturated fatty acid auxotropy for oleate. We therefore conducted a series of experiments indentical with those in

Figures 1 and 5 except that no oleate was added in phase 2. After starvation of cultures for 0.3 to 0.7 generation, gas chromatographic analysis showed that the oleate content had decreased to 45.9% and 34.2%, respectively. The spectra (not shown) of these samples were indistinguishable from those of Figure 5 and thus indicated that no significant (<10%) exchange of oleate occurred during oleate starvation.

Conclusion

The experiments successfully illustrate the ability of difference decoupling techniques to monitor exchange reactions in biosynthetic systems. Required sample quantities can be small (<5 mg) and satellites can be detected even in the presence of complex spectral lines.

The absence of significant unsaturated fatty acid exchange in *E. coli* indicates that the fatty acid composition of this organism is dictated by de novo synthesis and furthermore that experiments involving shifting of the fatty acid supplements required by fatty acid auxotrophs are not complicated by fatty acid exchange between phospholipid molecules.

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