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**THE EFFECTS OF TEMPERATURE AND FATTY ACID STRUCTURE
ON LIPID METABOLISM IN BACILLUS LICHENIFORMIS 9259**

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SUMMARY

Temperature of growth or incubation has, at most, subtle effects on the rate of incorporation of palmitate or 10-hexadecenoate into various lipids and on the final distribution of these fatty acids among the lipid classes in B. licheniformis 9259. However, temperature strongly affects the rate and extent of desaturation of palmitate to 10-hexadecenoate and to 5,10-hexadecadienoate and, in turn, the presence or absence of double bonds markedly influences further metabolism.

In growing cultures, desaturation of added [1-¹⁴C] palmitate was twice as great at 20° as at 35°. No diunsaturated acid was formed at 35° but was synthesized readily at 20°. Transfer of a culture from 35° to 20° had the same stimulatory effect on the rate of monodesaturation as did growth at 20°, but the rate of 5,10-hexadecadienoate formation in the transfer culture was only 10% of that in the culture growing at 20°.

At both 35° and 20° esterification of added

[1-¹⁴C] palmitate or (9,10-³H)-10-hexadecenoate to neutral lipid (1,2-diglyceride) was rapid. After initial esterification, however, neutral lipid activity steadily declined and phospholipid (chiefly phosphatidyl glycerol and phosphatidyl ethanolamine) increased at the expense of neutral lipid and at a rate related to fatty acid structure. Initial esterification to 1,2-diglyceride was more rapid for the unsaturated acid but palmitate activity was transferred more rapidly from neutral lipid to phospholipid.

Separation and phospholipase A hydrolysis of individual phospholipids from B. licheniformis showed that nC₁₆ fatty acids were concentrated in the 1-position of phosphatidyl ethanolamine and in the 2-position of phosphatidyl glycerol at both 35° and 20°, while anteiso C₁₅ was found almost exclusively in the 2-position of both phospholipids. Similar experiments were carried out on B. megaterium phospholipids and several differences between the two organisms in fatty acid distribution among and within

the phospholipids were noted.

INTRODUCTION

In many species and strains of Bacilli there is a pronounced effect of temperature on unsaturated fatty acid biosynthesis. In general, those Bacilli which can synthesize cis-5-monounsaturated fatty acids do so only at relatively low growth or incubation temperatures (20-25°). At higher temperatures (35-37°), little if any desaturation occurs¹⁻⁴. This relationship between temperature and desaturation is presumably part of an adaptive process which helps to maintain the functional integrity of the cell membrane in the face of a changing environmental temperature⁵⁻⁹. In Bacillus megaterium, which can desaturate in the 5-position at 20° but not at 35°, the inverse relationship between temperature and desaturation has been shown to result from the combined effects of temperature on the induction and stability of the desaturating enzyme system¹⁰⁻¹².

On the other hand, there are Bacilli strains which synthesize unsaturated fatty acids at both 35° and 20°, although usually a higher proportion of un-

saturated fatty acids is found at the lower temperature³. In general, these organisms produce monounsaturated fatty acids with the double bond located in the 8, 9 or 10 position. In the case of B. licheniformis 9259, cis-10-hexadecenoic acid is produced at both 35° and 20°, but in addition, a diunsaturated fatty acid (5,10-hexadecadienoic acid) is formed at 20°. The biosynthesis of this unique polyunsaturated fatty acid has already been described in some detail¹³⁻¹⁴. In the experiments reported here, we sought to determine, first of all, how temperature changes affect unsaturated fatty acid biosynthesis and the relative rate of incorporation of saturated and unsaturated fatty acids into various lipids. Secondly, we looked for temperature-related and structure-related differences in the distribution of various fatty acids in individual phospholipid components and in the distribution of these fatty acids in the 1 and 2 positions of the glycerol moiety of the phospholipids. The results show that temperature changes markedly affect unsaturated fatty acid

composition. To a lesser extent temperature also influences the way saturated and unsaturated fatty acids are utilized, but in this regard, the structure of the fatty acid itself is the more important determinate.

MATERIALS AND METHODS

Growth, incubation and harvesting of bacteria

B. licheniformis 9259 and B. megaterium 14581 were obtained from the American Type Culture Collection and were grown in constant-temperature rotatory incubator-shakers on a glucose-salts-casamino acid medium as previously described^{1,3,11}. Cells were harvested either by centrifugation or by passage of the culture medium through 0.8 μ m Millipore filters (Millipore Corp., Bedford, Mass., U.S.A.). Cells harvested by filtration were placed in the extraction solvent at once while cells obtained by centrifugation were sometimes frozen at -20° for work-up at a later time.

Substrates

[1-¹⁴C] Palmitic acid was obtained either from New England Nuclear Corp., Des Plaines, Ill., U.S.A.

or from Schwartz BioResearch Inc., Orangeburg, N.Y., U.S.A. and was added to the cultures as a solution in absolute ethanol. In general, syringes were employed to inject the substrate directly into the medium. [U-¹⁴C]-L-isoleucine (New England Nuclear Corp.) was added in water solution. [9,10-³H]-cis-10-hexadecenoic acid was isolated from B. licheniformis 9259 which had been grown at 35° in the presence of [9,10-³H] palmitic acid. The isolation and purification of this unsaturated substrate has been described in detail¹⁴.

Extraction of bacteria

After cells were harvested, the cell paste (and Millipore filter, if used) were homogenized with chloroform-methanol (2:1, v/v) in glass tissue grinders equipped with motor-driven teflon pestles. RADIN'S modification of the Folch extraction procedure¹⁵ was followed closely. After extraction, cell debris was removed by passing the extract through a glass column packed with glass wool and the effluent was washed with 0.2 volume of 0.04% calcium chloride solution. After the mixture settled into 2 phases, the upper water-

methanol phase was removed and the remaining chloroform phase, containing the lipids, was dried over anhydrous MgSO_4 and filtered. A second extraction procedure, that of VORBECK AND MARINETTI¹⁶ was also tested but the modified Folch procedure of Radin was generally used, since it is simpler and the extraction efficiency was almost identical for both methods.

Determination of free fatty acids

Lipid samples were analyzed for radioactive fatty acids by treating a portion of the extract, in methanol, with diazomethane in ether¹⁷ followed by silicic acid chromatography of the treated sample as previously described³. In this procedure, any free fatty acid in the lipid mixture is converted to the methyl ester which is eluted from silica gel with 3% ether in pentane. Simultaneous chromatography of an untreated lipid sample served as a control.

Conversion of lipid-bound fatty acids to methyl esters

To obtain methyl esters from either whole cells or isolated lipids, the samples were treated at 80° overnight in 5% HCl in anhydrous methanol as described

previously^{11,14}. The methyl esters were then extracted into pentane and purified by elution from silica gel with 3% ether in pentane³.

Separation of saturated from unsaturated methyl esters

Two different procedures were used. Generally, chromatography of the methyl esters on silica gel impregnated with AgNO_3 was employed¹⁴. This procedure separated the esters into saturated, monounsaturated and polyunsaturated fractions. At times, simple separation into saturated and unsaturated fractions was achieved by treatment of the methyl esters with mercuric acetate in methanol followed by silica gel chromatography of the products³.

Separation of individual lipids by thin-layer chromatography

The fractionation of Bacilli lipids into 3 components (phospholipids, neutral lipids, free fatty acids) was achieved by the method of SINGH et al.¹⁸. Precoated silica-gel plates, 5 x 20 cm in size with a layer thickness of 0.25 mm and obtained either from Mallinckrodt Chemical Works, St. Louis, Missouri,

U.S.A. (Chroma-Plate 76) or from EM Reagents Div., Brinkmann Instruments, Inc., Westbury, N.Y., U.S.A., were spotted with the lipid extract and developed in the solvent system acetone-petroleum ether, B.P. 30-60° (98:2, v/v). In this system the neutral lipids migrate close to the solvent front, the phospholipids remain near the origin and free fatty acids migrate between these two components. Lipid spots were located by placing the developed plates in an iodine chamber for several minutes. The location of radioactivity on the plates was determined by means of a radiochromatogram scanner (Model 7200, Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.). Areas under the scanning curve corresponding to each lipid class were cut from the graph paper and weighed to determine the distribution of radioactivity. At times, especially with low-activity samples, the individual lipid spots were scraped from the plates with a razor blade and then either counted directly in toluene scintillation solution or isolated by elution from the silica gel with chloroform-methanol-water

(10:5:1, v/v).

For resolution of individual neutral lipids or phospholipids by thin-layer chromatography, several other solvent systems were used and these are described in the "results" section.

Enzymatic hydrolysis of phospholipids

The positions of the fatty acids in the purified phospholipids were determined with the aid of phospholipase A of snake venom (dried venom of Crotalus adamanteus, Sigma Chemical Co., St. Louis, Missouri, U.S.A.). This phospholipase specifically hydrolyzes the 2-fatty ester linkage in phospholipids¹⁹. In a typical experiment, purified phospholipids were added with a 10-fold excess of a phospholipid standard as a carrier. Lipids were then dissolved in 2 ml of ether containing 0.01 ml of 1.5 M aqueous NH_4OH ²⁰. After mixing, 0.05 ml of venom solution was added. The venom solution contained 20 mg of venom per ml of 0.1 N Tris-chloride buffer, pH 7.2 and 0.01 M CaCl_2 . The mixtures were incubated, with gentle shaking, for 10 hours at room temperature. The reaction was

terminated by the addition of 2 ml of ethanol and the clear solution was evaporated to dryness under a stream of nitrogen. Controls, in which phospholipids were treated by the same procedure in the absence of snake venom showed little evidence of non-enzymatic hydrolysis.

The residue from the venom hydrolysis was taken up in chloroform and separated by silica gel thin-layer chromatography in the solvent system chloroform-methanol-water (65:35:4, v/v/v) and visualized in an iodine chamber. Three bands, corresponding to unreacted substrate, lysophosphatide and free fatty acid could be observed. The distribution of radioactivity was determined both by scanning the plate and by scraping and counting. Scintillation counting procedures were carried out as previously described¹¹. The standard lipids used as carriers or as reference compounds for thin-layer chromatography were obtained from Applied Science Laboratories, Inc., State College, Penna., U.S.A.

RESULTS

Incorporation of palmitate by *B. licheniformis* 9259

Recently published experiments³ with *B. megaterium* showed that this organism completely incorporated [1-¹⁴C] palmitate within 30 sec after its addition to the medium but that after 30 min, about 60% of the incorporated radioactivity had been excreted back into the medium as CO₂, β-hydroxy butyric acid and other water-soluble products derived from β-oxidation. There was little further excretion of [¹⁴C] products after 30 min and the activity remaining in the cells after this time could be recovered either as palmitate or its desaturation product, *cis*-5-hexadecenoate. *B. licheniformis* 9259 also takes up [1-¹⁴C]-palmitate rapidly from the medium but, unlike *B. megaterium*, does not excrete significant amounts of radioactivity after uptake. As Fig. 1 suggests, however, there is still some oxidation of incorporated [1-¹⁴C] palmitate. All of the added radioactivity can be recovered from the bacteria by HCl-catalyzed methanolysis of the cells (curve A) but only 70-80% of this activity is soluble in pentane

Fig. 1

(curve B). Most of the pentane-soluble material can be eluted from silica gel with 2% ether in pentane (curve C). This solvent will readily elute methyl palmitate and its unsaturated derivatives but will leave more polar constituents (such as the methyl esters of β -hydroxy acids) on the column. The remaining activity can be eluted with 20% ether in pentane and consists chiefly of methyl- β -hydroxy-palmitate. Presumably, the difference between the counts present in the methanolysis mixture (curve A) and those extracted into pentane (curve B) represent short chain, relatively pentane-insoluble oxidation products such as methyl- β -hydroxybutyrate. The total radioactivity recovered in the chloroform phase after Folch extraction is only about half of that obtained by direct methanolysis but all of this activity can be recovered, after methanolysis of the extracted material, by extraction into pentane (curve D). Most of this activity, in turn, can be eluted from silica gel with 2% ether in pentane (curve E). The incorporation of radioactivity from [1- ^{14}C] palmitate was also studied

at 35° using the same procedures as described for 20° and the results did not differ significantly.

Esterification of palmitate

In order to determine the proportion of [^{14}C] activity recovered by extraction which represented actual uptake and esterification of exogenous palmitate as opposed to either physical adsorption on the cell surface or passive diffusion into a metabolically inactive pool within the cell, the free fatty acid content of the cell extracts obtained by the modified Folch and the Vorbeck-Marinetti procedures was determined. As Fig. 2 shows, esterification is relatively rapid (about 83% complete after 30 min) and reaches a constant level of about 96% after 2 hours of incubation.

Fig. 2

Desaturation of palmitate

Table I

Table I shows the rate of conversion of [$1\text{-}^{14}\text{C}$] palmitate to monounsaturated and diunsaturated fatty acids during the course of incubation and compares the relative yields of these derivatives from cells either treated directly with HCl-methanol to obtain

the methyl esters or else first extracted by the modified Folch or the Vorbeck-Marinetti procedures to obtain the lipids which were then transesterified. All three procedures gave similar results but the two extraction procedures gave slightly higher relative yields of unsaturated fatty acids throughout the course of the incubation. This difference was particularly evident in the monounsaturated fractions for the later incubation periods.

Incorporation of radioactivity for [1-¹⁴C] palmitate and [9,10-³H]-10-hexadecenoate into neutral and phospholipids at 20° and 35°

Figs. 3 & 4

Figs. 3 and 4 show the disappearance of free [1-¹⁴C] palmitic acid and the incorporation of ¹⁴C-label into neutral and phospholipids at 35° and 20°

Tables II & III

respectively, while Tables II and III show the relative distribution of ¹⁴C-activity in the unsaturated acids derived from each of these lipids during the course of incubation with [1-¹⁴C] palmitate. A similar experiment was carried out using [9,10-³H]-10-hexadecenoic acid at 35° and 20° and these results

Figs. 5 & 6

are shown in Figs. 5 and 6.

Identification of neutral lipid and phospholipid components in *B. licheniformis* 9259

The identity of the neutral lipids and phospholipids was determined after incubation of the cultures at either 35° or 20° in the presence of [1-¹⁴C]

Fig. 7

palmitate as described under Figs. 3 and 4. Fig. 7

shows the results which led to the identification of the major neutral lipid component as 1,2-diglyceride

Fig. 8

while Fig. 8 describes the identification of the 2 major phospholipids. Several minor components were present in both the neutral lipid and phospholipid fractions but they were not rigorously characterized. Tentative identifications of monoglyceride, phosphatidic acid and diphosphatidyl glycerol were made on the basis of chromatographic behavior but other tests were not carried out on these components.

Distribution of radioactivity in the phospholipids

Table IV

Table IV shows the relative distribution of fatty acid radioactivity in the 1 and 2 positions of phosphatidyl ethanolamine and phosphatidyl glycerol

in both B. licheniformis 9259 and, for comparison purposes, in B. megaterium 14581, when these organisms were grown at several temperatures in the presence of either $[1-^{14}\text{C}]$ palmitate or $[\text{U}-^{14}\text{C}]\text{-L-}$ isoleucine.

DISCUSSION

Although the experiments shown in Figs. 1 and 2 and in Table I were designed primarily to determine the validity of the methods used in this study, they also elucidate certain basic aspects of fatty acid metabolism in B. licheniformis 9259. As Fig. 1 shows, exogenous $[1-^{14}\text{C}]$ palmitate is rapidly taken up by B. licheniformis. Essentially all of the radioactivity added to the medium can be recovered in the bacteria after 30 min (curve A, Fig. 1). As Fig. 2 shows, this radioactivity cannot be ascribed to passive adsorption of $[1-^{14}\text{C}]$ palmitate on the cell surface, since esterification takes place almost as rapidly as uptake. In this respect, B. licheniformis and B. megaterium are similar, although utilization of palmitate by B. megaterium is even more rapid¹¹.

In both organisms part of the palmitate is oxidized during the uptake period but thereafter, it seems impervious to further oxidation. B. megaterium, however, excretes the oxidation products back into the medium as CO₂ and water-soluble fragments while B. licheniformis retains the oxidation products as β -hydroxy-palmitate and, presumably poly- β -hydroxy-butyrate.

Table I, which shows the rate of conversion of [1-¹⁴C] palmitate to unsaturated fatty acids in B. licheniformis at 20° is quite revealing, especially when compared with similar data shown in Table III. In the experiment shown in Table III, B. licheniformis was grown at 35° and transferred to 20° just 30 min before the addition of [1-¹⁴C] palmitate to the medium. In the experiment whose results are shown in Table I, [1-¹⁴C] palmitate was added to a culture of B. licheniformis which had been grown at 20° from inoculum. In both cultures, the rate of conversion of palmitate to unsaturated fatty acids is essentially the same and reaches the same level after 8-9 hours

(85-87° desaturation). However, in the culture growing at 20° (Table I), the rate of synthesis of the diunsaturated fatty acid (5,10-hexadecadienoate) is more than 10 times greater than in the culture transferred to 20° (Table III). Since the diunsaturated fatty acid is necessarily derived from the monounsaturated precursors¹⁴ and hence reflects monounsaturated fatty acid synthesis, the rates of conversion of [1-¹⁴C] palmitate to monounsaturated fatty acids are the same in both cultures but further conversion to the diunsaturated derivative is severely retarded in the transferred culture. In interpreting these observations, it should be remembered that Δ^{10} -desaturation proceeds at either 35° or 20° but that the formation of 5,10-hexadecadienoic acid occurs only at the lower temperature¹⁴. This fact suggests that the Δ^5 -desaturase is not synthesized or is unstable at 35° and is thus similar in this respect to the Δ^5 -desaturase of B. megaterium^{3,12}. However, in B. megaterium, when a culture grown at 35° is transferred to 20°, hyperinduction of the Δ^5 -desaturase occurs;

that is, the induction of desaturating enzyme levels in cultures transferred from 35° to 20° are well above levels of the same enzyme found in comparable cultures grown only at 20°¹⁰. Just the opposite effect is observed in B. licheniformis. We think that the explanation lies in the fact that B. licheniformis is much more susceptible to cold shock than is B. megaterium and undergoes a much greater and longer-lasting depression in the rate of protein synthesis after transfer from 35° to 20° (D. FUJI AND A. FULCO, unpublished). Thus, any propensity for hyperinduction of the Δ^5 -desaturase in B. licheniformis is more than canceled by the cold-induced depression of protein synthesis. On the other hand, the level of Δ^{10} -desaturase, which is already present at 35°, should not be immediately affected by transfer to 20° and indeed, might remain at a high level for a considerable period of time, even in the face of reduced protein synthesis, if its stability is increased at the lower temperature. Thus, it would seem that di-unsaturated fatty acid biosynthesis plays a relatively

unimportant role in the rapid adaptation of B. licheniformis to a sudden change to a lower temperature. On the other hand, in cultures growing at 20°, the biosynthesis of 5,10-hexadecadienoic acid may be very important in maintaining optimal membrane function.

Previously published data¹⁴ as well as the data in Tables III and IV show quite clearly that the conversion of exogenously supplied radioactive palmitic acid to 10-hexadecenoic acid is always high in cultures either growing at 20° or incubated at 20° after transfer from 35°. This high rate of conversion is also reflected in the endogenous fatty acid composition of B. licheniformis cultures grown or incubated at 20°. Thus, in B. licheniformis grown into early log phase at 35° and then incubated at 20° for 8 hours, 91% of the endogenous nC₁₆ was found to be unsaturated as compared to only 25% in the same culture maintained at 35° throughout the course of the experiment¹⁴. At 35°, the amount of added radioactive palmitate converted to 10-hexadecenoate is always

lower than in comparable cultures at 20° (Tables II and IV) but may still exceed 50%. However, the rate and amount of conversion of added palmitate at 35° is very dependent on the growth stage of the culture at the time the substrate is supplied. Early log-phase cultures (Table IV) are more active in this respect than mid log-phase cultures (Table II) while late log-phase cultures convert little, if any, added [1-¹⁴C] palmitate to unsaturated acids (T. PARKER, N. CHANG AND A. FULCO, unpublished). It would seem that the very high rate of conversion of palmitate to 10-hexadecenoate at 20° reflects a need for this product in the adaptation of B. licheniformis to low temperatures, although perhaps in a secondary role as the precursor of 5,10-hexadecadienoic acid. At 35°, the rate of synthesis of 10-hexadecenoic acid seems to be roughly proportional to growth rate and one would assume that, even at this higher temperature, the monounsaturated acid plays some role in maintaining optimal membrane function.

The studies on the uptake and incorporation into

lipids of palmitate and 10-hexadecenoate at 35° and 20° reveal similar patterns at both temperatures and with both substrates (Figs. 3-6). In all cases, esterification of the substrate is rapid, neutral lipid activity increases rapidly and then declines to a low level and phospholipid activity increases quite rapidly during the first few minutes and then much less rapidly but steadily with increasing incubation time. Initially, neutral lipid and phospholipid seem to be synthesized independently, while at later times, after esterification is complete, there is an obvious reciprocal relationship between neutral lipid and phospholipid activity, with the flow of ¹⁴C-label proceeding from neutral lipid to phospholipid. The major neutral lipid component proved to be 1,2-diglyceride (Fig. 7) while the phospholipid is a mixture of phosphatidyl ethanolamine and phosphatidyl ~~glycerol~~ ²¹ ~~choline~~ (Fig. 8). PATTERSON AND LENNARZ have shown, in cell-free preparations from Bacillus megaterium, that these phospholipids are formed in the expected way from phosphatidic acid via CDP-diglyceride.

Phosphatidic acid is also the usual precursor for 1,2-diglyceride^{22,23}. The 1,2-diglyceride is formed directly by hydrolysis of the phosphate group and has been found as a major constituent in *Bacilli*²⁴⁻²⁶.

We feel that these common pathways are also operating in *B. licheniformis* and that, initially, labeled palmitate and 10-hexadecenoate are incorporated into a small metabolically active pool of phosphatidic acid which is either converted to diglyceride by hydrolysis or to phospholipid via CDP-diglyceride. Once all of the radioactive substrate is incorporated however, a mechanism must exist for the more gradual transfer of label from diglyceride to phospholipid. This could take place by phosphorylation of diglyceride to reform phosphatidic acid and then conversion of this product to phospholipid in the usual way, by transacylation, by hydrolysis of the diglyceride acyl groups followed by their reincorporation into phospholipids, or by a combination of these mechanisms.

Although the overall patterns of label incorporation are similar for both palmitate and 10-

hexadecenoate at 35° and 20°, there are distinct differences which could be ascribed to either temperature effects or to the nature of the substrate. For example, palmitate esterification is more rapid at 35° (Fig. 3) than at 20° (Fig. 4) and the transfer of label from neutral lipids to phospholipids is much more rapid. Thus, after 1.5 hours at 35°, phospholipids contain about 78% of the ^{14}C -activity while at 20°, only 40% of the radioactivity is found in the phospholipids at this time period. After 3 hours at 35°, a steady state is reached in which about 88-90% of the activity is in the phospholipids, 7-8% in the neutral lipids and 4-5% in free fatty acids. At 20°, a steady state is not reached until 6 hours; after this time, phospholipids contain about 77% of the activity, neutral lipids about 20% and free fatty acids, 3%. At first glance, this would seem to reflect a direct effect of temperature on the utilization of palmitate. However, one must remember that at 35°, desaturation of palmitate is low and terminates after 1.5 hours (Table II), while at 20°,

desaturation is very rapid (60% at 1.5 hours and close to 85% by 9 hours). Thus, if C₁₆ unsaturated fatty acids were incorporated more slowly than palmitate into phospholipids, the difference in incorporation of radioactivity into phospholipids could result primarily from the in vivo alteration of substrate rather than from a change in the rate of incorporation arising from a direct temperature effect. That this is indeed the situation is clearly suggested by the results of 10-hexadecenoate incorporation at 35° and 20° (Figs. 5 and 6). The esterification of 10-hexadecenoic acid is more rapid than esterification of palmitate at both temperatures and most of the radioactivity appears first in the neutral lipids. After the first 20-30 minutes, at both 35° and 20°, the neutral lipid radioactivity derived from 10-hexadecenoate has peaked and starts to decrease as phospholipid activity increases. When palmitate incorporation is compared with 10-hexadecenoate incorporation at 35°, it is apparent that the transfer of radioactivity from neutral lipids to

phospholipids is much more rapid for palmitate. At 20°, the initial rate of incorporation of radioactivity into phospholipids is more rapid in the cells incubated with palmitate but after 1.5 hours (when most of the palmitate has been converted to monounsaturated fatty acid) the rate of transfer of radioactivity from neutral lipids to phospholipids is about the same in both cultures. Thus, it appears that the initial rate of incorporation of a fatty acid as well as the rate of transfer of activity from neutral lipids to phospholipids is more dependent on the structure of the fatty acid than on the temperature of incubation. We can state with some confidence that exogenous 10-hexadecenoic acid is esterified more rapidly than palmitate at both temperatures and is converted more rapidly to neutral lipids. Palmitate, on the other hand is initially converted more rapidly to phospholipids and, at 35°, at least, is transferred much more rapidly from neutral lipids to phospholipids. One direct temperature effect on incorporation can be discerned,

however. When Figs. 5 and 6 are compared, it can be seen that the transfer of 10-hexadecenoate radioactivity from neutral lipids to phospholipids proceeds more rapidly at 20° than at 35°. Temperature may also affect, to some extent, the relative rate of incorporation of unsaturated fatty acids into phospholipids and neutral lipids (compare Tables II and III) but the differences are small and they diminish with time.

When the phospholipids of cultures of B. licheniformis grown in the presence of various radioactive substrates at either 35° or 20° are separated and the relative distribution of radioactivity is determined for both the intact phospholipids and for the 1 and 2 positions of each phospholipid, differences in the relative distribution of radioactivity are observed (Table IV, Expts. 1-3). Similar experiments with B. megaterium (Table IV, Expts. 4-6) also show differences which can be ascribed primarily to differences in substrate. In B. licheniformis grown at 35° in the presence of [1-¹⁴C] palmitate, about half of the substrate had been desaturated by late

log phase and phosphatidyl ethanolamine contained about 56% of the total phospholipid activity (Expt. 1). In turn, about 2/3 of the phosphatidyl ethanolamine activity was located in the 1-position. At 20°, 86% of the palmitate was desaturated and 70% of the total phospholipid radioactivity was located in phosphatidyl ethanolamine, again with the 1-position being favored over the 2-position but not quite as strongly as at 20°. Clearly, in B. licheniformis, the data show that the nC₁₆ fatty acid (saturated or unsaturated) is preferentially incorporated into phosphatidyl ethanolamine rather than into phosphatidyl glycerol and, in phosphatidyl ethanolamine, the 1-position is preferred. Since the ratio of saturated to unsaturated fatty acids in each position was not determined, absolute specificities cannot be given. However, an increase in unsaturated fatty acid from 48% (Expt. 1) to 86% (Expt. 2) further increases the percentage of radioactivity in phosphatidyl ethanolamine (from 56% to 70%) and also slightly decreases the amount of radioactivity in the 1-position of

phosphatidyl ethanolamine relative to the 2-position. This same relative decrease in the activity incorporated into the 1-position with increased desaturation is also observed in phosphatidyl glycerol. From this, we can conclude that there is some preference for incorporation of C₁₆ unsaturated fatty acid into phosphatidyl ethanolamine rather than into phosphatidyl glycerol and that, within each phospholipid, the ratio of unsaturated to saturated fatty acid at the 2-position may be higher than at the 1-position but this specificity is not strong.

In Expt. 3, where [U-¹⁴C]-L-isoleucine was incubated with B. licheniformis at 35°, the pattern of label incorporation is radically different from that arising from palmitate. The label is distributed more evenly among phosphatidyl ethanolamine, phosphatidyl glycerol and other unidentified phospholipids, with phosphatidyl glycerol being favored. The striking difference, however is that almost all of the radioactivity of both identified phospholipids is found in the 2-position (90% for phosphatidyl glycerol, 85%

for phosphatidyl ethanolamine). We have found, when [U-¹⁴C]-L-isoleucine is incubated with B.

licheniformis at 35°, more than 90% of the radioactivity is incorporated into the anteiso-C₁₅ acid, 12-methyltetradecanoate (J. QUINT AND A. FULCO, unpublished). Therefore, we can conclude that this fatty acid is esterified almost exclusively into the 2-position of the major phospholipids of B. licheniformis. This is in complete agreement with the results obtained recently by KANEDA²⁷ who found that anteiso-C₁₅ was esterified chiefly into the 2-position of both phosphatidyl ethanolamine and phosphatidyl glycerol in B. subtilis.

The experiments with B. megaterium (Table IV, Expts. 4-6) were designed for direct comparison with those carried out on B. licheniformis. Indeed, since there is essentially no unsaturated fatty acid biosynthesis at 35° in B. megaterium (Expt. 4) and since unsaturated fatty acid content was determined for each position in phosphatidyl ethanolamine and phosphatidyl glycerol in the 20° experiment (Expt. 5), esterifica-

tion specificities could be determined with considerable accuracy. One notable difference between B. licheniformis and B. megaterium is found in the distribution of radioactivity between phosphatidyl ethanolamine and phosphatidyl glycerol. Unlike B. licheniformis, B. megaterium incorporates more radioactivity into phosphatidyl glycerol at both temperatures. The difference is especially notable at 20° (compare Expt. 2 with Expt. 5). A second difference can also be seen in this comparison; at 20°, the 2-position in phosphatidyl ethanolamine is slightly favored in B. megaterium while the opposite is true in B. licheniformis. Otherwise, the patterns in the label distribution in the phospholipids of the two organisms are quite similar. In B. megaterium, palmitate is esterified predominantly into the 2-position of phosphatidyl glycerol and the 1-position of phosphatidyl ethanolamine at both temperatures while the unsaturated acid (5-hexadecenoate) is found chiefly in the 2-position of phosphatidyl ethanolamine and phosphatidyl glycerol. The ratio of un-

saturated to saturated fatty acid is somewhat higher in the 2-position in both phospholipids, a result that was also inferred for B. licheniformis. As in B. licheniformis, almost all of the radioactivity derived from [U-¹⁴C]-L-isoleucine is found in the 2-position of both phospholipids.

It would be useful, at this point, to compare our results obtained in B. licheniformis and B. megaterium with those obtained by KANEDA in B. subtilis²⁷. In all three organisms, anteiso-C₁₅ is found chiefly in the 2-position of both phosphatidyl ethanolamine and phosphatidyl glycerol. We had shown previously¹⁴ that, as 35°, the major branched-chain fatty acids of B. licheniformis are C₁₅ and C₁₇. In this organism, the major C₁₅ isomer is anteiso (J. QUINT AND A. FULCO, unpublished) just as in B. subtilis. Thus, the branched C₁₇ acids must be located chiefly in the 1-position of the phospholipids. This too, is in good agreement with KANEDA'S findings in B. subtilis. KANEDA found that palmitate was located chiefly in the 1-position of both major

phospholipids in B. subtilis while we found that, in B. megaterium and probably in B. licheniformis as well, nC_{16} was located primarily in the 1-position of phosphatidyl ethanolamine but in the 2-position of phosphatidyl glycerol. These difference can probably be attributed to biological variations among the 3 species studied rather than to variations arising from different experimental conditions.

In view of the results discussed here and in an earlier paper¹⁴, it would seem that B. licheniformis adapts to a lower environmental temperature primarily by changing its fatty acid composition rather than by altering either the rate of incorporation of various fatty acids into lipids (a mechanism recently found by SINENSKY²⁸ to operate in E. coli) or by drastically altering the ratios of phospholipid components. There are small changes both in the rate of incorporation of unsaturated fatty acids and in the ratios of phosphatidyl ethanolamine to phosphatidyl glycerol which do seem to be mediated by temperature but they are probably not of major importance. On the other

hand, the biosynthesis of a unique polyunsaturated fatty acid coupled with a relative increase in mono-unsaturated C₁₆ and a relative decrease in palmitate and branched C₁₇¹⁴ are probably of great significance in the adaptation of B. licheniformis to a low growth or incubation temperature.

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REFERENCES

- 1 Fulco, A.J., Levy, R. and Bloch, K. (1964) J. Biol. Chem. 239, 998-1003.
- 2 Fulco, A.J. (1967) Biochim. Biophys. Acta 744, 701-703.
- 3 Fulco, A.J. (1969) J. Biol. Chem. 244, 889-895.
- 4 Kaneda, T. (1971) Biochem. Biophys. Res. Comm. 43, 298-301.
- 5 Wilson, G., Rose, S.P. and Fox, C.F. (1970) Biochem. Biophys. Res. Comm. 38, 617-623.
- 6 Rose, A.H. (1968) J. Appl. Bacteriol. 31, 1-11.
- 7 Smith, P.F. (1969) Lipids 4, 331-336.
- 8 Schairer, H.U. and Overath, P. (1969) J. Mol. Biol. 44, 209-214.
- 9 Haest, C.W.M., DeGier, J. and Van Deenen, L.L.M. (1969) Chem. Phys. Lipids 3, 413-417.
- 10 Fulco, A.J. (1970) Biochim. Biophys. Acta 218, 558-560.
- 11 Fulco, A.J. (1972) J. Biol. Chem. 247, 3503-3510.
- 12 Fulco, A.J. (1972) J. Biol. Chem. 247, 3511-3519.
- 13 Fulco, A.J. (1969) Biochim. Biophys. Acta 187,

169-171.

- 14 Fulco, A.J. (1970) J. Biol. Chem. 245, 2985-2990.
- 15 Radin, N. (1969) in Methods in Enzymology
(Lowenstein, J.M., ed), Vol. XIV, pp. 246-249,
Academic Press, New York.
- 16 Vorbeck, M.L. and Marinetti, G.V. (1965) J. Lipid
Res. 66, 3-6.
- 17 Schlenk, H. and Gellerman, J.L. (1960) Anal. Chem.
32, 1412-1414.
- 18 Singh, E.J., Gershbein, L.L. and O'Neill, H.J.
(1966) Lipids 1, 274-278.
- 19 Van Deenen, L.L.M. and de Haas, G.H. (1963)
Biochim. Biophys. Acta 70, 538-553.
- 20 Hildebrand, J.G. and Law, J.H. (1964) Biochemistry
3, 1304-1308.
- 21 Patterson, P.H. and Lennarz, W.J. (1971) J. Biol.
Chem. 246, 1062-1072.
- 22 Gurr, M.I. and James, A.T. (1971) Lipid Biochem-
chemistry, p. 94, Cornell University Press,
Ithaca, New York.
- 23 Hitchcock, C. and Nichols, B.W. (1971) Plant Lipid

Biochemistry, p. 178, Academic Press, London and New York.

- 24 Kates, M., Kushner, D.J. and James, A.T. (1962)
Can. J. Biochem. Physiol. 40, 83-93.
- 25 Lillich, T.L. and White, D.C. (1971) J. Bacteriol.
107, 790-797.
- 26 Beebe, J.L. (1971) J. Bacteriol. 107, 704-711.
- 27 Kaneda, T. (1972) Biochim. Biophys. Acta 270,
32-39.
- 28 Sinensky, M. (1971) J. Bacteriol. 106, 449-458.

LEGENDS

Fig. 1. Incorporation of radioactivity from $[1-^{14}\text{C}]$ palmitate at 20° . B. licheniformis was grown at 20° to mid-log phase (2.8 g/l) and $[1-^{14}\text{C}]$ palmitic acid was then added at a concentration of 1 $\mu\text{mole/l}$ of medium. At the designated intervals, 10 ml portions of the culture were applied to Millipore filters in triplicate and the filter discs containing the harvested cells were then assayed for radioactivity by several methods. One series of samples was treated directly with HCl-methanol and a portion of each transesterification mixture was taken for counting (curve A). The transesterification mixtures were then extracted with pentane and portions of the pentane extracts were counted (curve B). Finally, the pentane-soluble material was chromatographed on silica gel and the unsubstituted methyl esters were eluted with 3% ether in pentane and counted (curve C). A second series of samples were extracted with 2:1 chloroform-methanol by Radin's modification of the Folch procedure while the third series was preheated at 65° for 5 min in methanol before chloroform was added

(Vorbeck-Marinetti procedure). Preheating with methanol did not significantly alter the recovery of radioactivity at any stage of the work-up and hence, although the second and third series of samples were analyzed separately, the counting results at each time interval were averaged and are expressed together. The extracts were reduced to constant volume under N_2 , the resulting lipid residues were transesterified and the methanolysis mixtures were extracted with pentane in the usual manner. Although samples were taken for counting at each stage of the procedure, there were no significant differences between the counts recovered in the pentane-soluble material (curve D) and in the chloroform-methanol extract or the transesterification mixture. Curve E represents the pentane-soluble material that was eluted from silica-gel with 3% ether in pentane.

Fig. 2. Esterification of [$1-^{14}C$] palmitate at $20^\circ C$. Portions of each lipid extract of B. licheniformis obtained from the experiment described in the legend for Fig. 1 were assayed for ^{14}C -labeled free fatty

acids by the diazomethane-silica gel chromatography procedure. Fig. 2 shows the free fatty acid content of the extract obtained by the Radin-modified Folch extraction (curve A) and by the Vorbeck-Marinetti procedure (curve B).

Fig. 3. Incorporation of radioactivity from $[1-^{14}\text{C}]$ palmitate into neutral and phospholipids at 35° . B. licheniformis was grown into mid-log phase (3.2 g/l) at 35° and $[1-^{14}\text{C}]$ palmitic acid was added at a concentration of 1 $\mu\text{mole/l}$ of medium. Samples were then harvested by filtration at the times indicated and extracted by the modified Folch procedure. Portions of the extracted lipids were then separated by silica gel thin-layer chromatography into 3 fractions (neutral lipids, free fatty acids and phospholipids) in the system acetone-petroleum ether, (98:2, v/v). The spots were visualized by iodine vapor and the distribution of radioactivity on the plates was determined by radioscanning. Finally, the radioactive spots corresponding to the various lipid fractions were scraped from the plates and

the radioactivity determined by scintillation counting. In Fig. 3, above, phospholipid activity is given by curve A, neutral lipid activity by curve B and free fatty acid activity by curve C.

Fig. 4. Incorporation of radioactivity from $[1-^{14}\text{C}]$ palmitate into neutral and phospholipids at 20° . B. licheniformis, grown into mid-log phase at 35° (see legend for Fig. 3), was transferred to 20° and incubated at this temperature for 30 min before the addition of $[1-^{14}\text{C}]$ palmitate ($1\ \mu\text{mole/l}$). Incubation was then continued at 20° for 9 h. All other conditions were as described for Fig. 3. Radioactivity recovered in each lipid fraction is given by curve A (phospholipids), curve B (neutral lipids) and curve C (free fatty acids).

Fig. 5. Incorporation of radioactivity from $[9,10-^3\text{H}]-10$ -hexadecenoate into neutral and phospholipids at 35° . The experiment was carried out as described under Fig. 3 except that $[9,10-^3\text{H}]-10$ -hexadecenoic acid ($1\ \mu\text{mole/l}$) was used as a substrate. Curve A, phospholipids; Curve B, neutral lipids; Curve C, free fatty acids.

Fig. 6. Incorporation of radioactivity from [9,10-³H]-10-hexadecenoate into neutral and phospholipids at 20°. This experiment was carried out as described under Fig. 4 except that [9,10-³H]-10-hexadecenoic acid was used as substrate. Curve A, phospholipids; Curve B, neutral lipids; Curve C, free fatty acids.

Fig. 7. Identification of the radioactive neutral lipid component of B. licheniformis formed during incubation of the cells with [1-¹⁴C] palmitate. B. licheniformis was incubated at 35° with [1-¹⁴C] palmitic acid and the lipids were isolated as described in Fig. 3. Phospholipids were separated from free fatty acids and neutral lipids by preparative thin-layer chromatography on silica gel with acetone-petroleum ether, (98:2, v/v). All material migrating above the phospholipid components was scraped from the plate and recovered from the silica gel by extraction with chloroform-methanol-water (10:5:1, v/v/v). This material was then divided into 2 portions, one of which was treated with diazomethane to convert free fatty acid to the methyl ester and the second of

which remained untreated. Samples were then applied, along with appropriate standards, onto silica gel plates and developed for 75 min in benzene-ether-ethyl acetate-acetic acid (80:10:10:0.2, by vol.). In one experiment, the diazomethane-treated sample and the untreated sample were spotted on separate plates and each plate was scanned for radioactivity after development. Scan 1 (above) was obtained from the untreated sample while scan 2 was obtained from the sample treated with diazomethane. The treated and untreated samples and the standard mixture were also chromatographed together on the same plate and the components were visualized by iodine vapor. This plate is reproduced above; A, untreated sample; B, diazomethane-treated sample. Chemical tests of the B. licheniformis lipid corresponding to 1,2-diglyceride were identical to those conducted on an authentic sample of 1,2-dipalmitin. The tests for phosphorus and nitrogen were negative and basic hydrolysis yielded only free fatty acid and glycerol.

Fig. 8. Identification of the radioactive phospholipid components of B. licheniformis formed during incubation of the cells with [1-¹⁴C] palmitate. The incubation and isolation of the lipids are described in Fig. 7. The phospholipid components were separated from other lipids by thin-layer chromatography on silica gel with acetone-petroleum ether (98:2, v/v), scraped from the plate and recovered by extraction of the silica gel with chloroform-methanol-water (10:5:1, v/v). This material was chromatographed for 45 min on a silica gel plate in chloroform-methanol-acetic acid-water (80:10:2:0.75, by vol.) and the plate was then scanned for radioactivity. About 95% of the ¹⁴C-activity was located in two spots which were not clearly separated from each other. These spots were carefully eluted from the plate and rechromatographed on silica gel for 130 min using chloroform-methanol-water (65:35:4, v/v/v). This solvent system separates phosphatidyl ethanolamine from phosphatidyl glycerol. The spots were then visualized with iodine vapor and scanned for radioactivity with the results shown above. Both radioactivity and mass corresponded with authentic

samples of phosphatidyl ethanolamine and phosphatidyl glycerol. Correspondence was also obtained using other solvent systems on silica gel G, including chloroform-methanol-30% ammonia-water (70:25:3.5:1.5, by vol.), and on silica gel H using 2 solvent systems sequentially. In addition, chemical tests on the lipid corresponding to phosphatidyl ethanolamine and on an authentic sample gave identical results (i.e. positive ninhydrin reaction, identical hydrolysis products and essentially the same phosphorus ratios). An authentic sample of phosphatidyl glycerol and the lipid corresponding to phosphatidyl glycerol (but obtained from B. licheniformis) both gave a positive reaction with periodate-Schiff reagent and a negative reaction with ninhydrin. Mild basic hydrolysis also produced products which behaved identically on thin-layer plates developed in both polar and non-polar solvent systems.

TABLE I

DESATURATION OF [1-¹⁴C] PALMITATE AT 20°

Portions of the unsubstituted methyl esters obtained from the experiments described in the legend for Fig. 1 were separated into saturated, monounsaturated and diunsaturated fractions by AgNO₃-silica gel chromatography and the radioactivity in each fraction was determined by scintillation counting. The results are grouped according to the procedure used to obtain or extract the bacterial lipids (see also Fig. 1).

	% Distribution of ¹⁴ C in unsubstituted methyl esters								
	Direct HCl-CH ₃ OH treatment			Radin-modified Folch extraction			Vorbeck-Marinetti extraction		
	16:0	16:1	16:2	16:0	16:1	16:2	16:0	16:1	16:2
0.25	95.0	4.8	0.2	94.4	5.3	0.3	94.5	5.3	0.2
0.50	85.2	14.1	0.7	81.6	17.8	0.6	77.7	21.5	0.8
1.00	62.4	35.4	2.2	58.7	38.5	2.8	53.7	44.6	1.7
1.50	44.9	50.9	4.2	40.6	53.6	5.8	46.1	50.3	3.6
2.00	34.4	58.3	7.3	33.7	58.5	7.8	29.6	60.3	10.1
2.75	30.8	59.3	9.9	26.2	63.7	10.1	24.7	62.6	12.7
3.50	26.3	61.6	12.1	20.4	64.9	14.7	21.7	62.1	16.2
4.50	22.2	62.4	15.4	18.3	61.7	20.0	16.3	64.9	18.8
6.00	18.8	62.2	19.0	16.8	59.9	23.2	15.9	61.5	22.6
8.00	18.3	60.4	21.3	12.7	60.2	27.1	12.5	60.4	27.1

TABLE II

PERCENT DISTRIBUTION OF RADIOACTIVITY IN UNSATURATED FATTY ACIDS IN VARIOUS LIPID FRACTIONS DURING INCUBATION OF B. LICHENIFORMIS WITH [1-¹⁴C] PALMITATE AT 35°

Portions of the extracted lipids from the experiment described under Fig. 3 were separated by thin-layer chromatography as described. The distribution of radioactivity on the plates was determined by scanning and the radioactive spots corresponding to neutral lipids, phospholipids and free fatty acids were scraped from the plates, extracted and transesterified with HCl-methanol. The methyl esters were separated into saturated and unsaturated fractions by AgNO₃-silica gel chromatography. Since there is no detectable biosynthesis of polyunsaturated fatty acids at 35°, the unsaturated fraction represents essentially monounsaturated (10-hexadecenoic) acid.

Incubation time (hours)	Phospholipid unsaturated fatty acids (%)	Neutral lipid unsaturated fatty acids (%)	Free unsaturated fatty acids (%)
0.5	3.6	3.9	2.2
1.0	8.4	8.9	4.6
1.5	15.5	14.0	9.8
2	17.7	14.8	12.5
3	15.4	13.2	9.9
4	15.9	11.9	9.4
5	11.4	12.1	10.2
6	12.7	13.0	9.7
9	15.2	15.3	8.3

TABLE III

PERCENT DISTRIBUTION OF RADIOACTIVITY IN SATURATED, MONOUNSATURATED, AND POLYUNSATURATED FATTY ACIDS IN VARIOUS LIPID FRACTIONS DURING INCUBATION OF B. LICHENIFORMIS WITH [1-¹⁴C] PALMITATE AT 20°

The procedure used is described in Table II.

Incubation time (hours)	Phospholipid fatty acids (%)			Neutral lipid fatty acids (%)			Free fatty acids (%)		
	16:0	16:1	16:2	16:0	16:1	16:2	16:0	16:1	16:2
0.10	85.2	14.1	0.6	88.8	10.5	0.7	98.9	0.7	0.4
0.25	80.0	19.3	0.6	86.4	13.0	0.6	98.8	0.8	0.4
0.50	75.8	23.5	0.7	82.2	17.2	0.6	97.2	2.4	0.4
0.75	63.7	35.2	1.1	75.3	23.8	0.8	83.8	15.2	1.0
1.00	51.5	47.0	1.6	65.0	33.9	1.1	73.1	25.9	1.0
1.50	35.6	62.6	1.9	42.4	56.0	1.7	48.3	50.0	1.7
2.00	20.9	77.4	1.8	25.5	72.7	1.8	32.9	65.4	1.7
3.00	23.1	74.9	2.0	19.1	79.2	1.7	37.9	60.0	2.1
4.50	16.9	80.9	2.2	17.6	80.7	1.7	35.5	64.0	2.5
6.50	16.1	81.7	2.2	17.2	80.8	2.0	30.0	67.0	3.0
9.00	14.8	82.9	2.3	17.1	80.5	2.4	24.6	70.5	4.9

TABLE IV

DISTRIBUTION OF RADIOACTIVITY IN PHOSPHOLIPIDS OF B. LICHENIFORMIS AND B. MEGATERIUM GROWN AT 35° OR 20° WITH [1-¹⁴C] PALMITATE OR [U-¹⁴C]-L-ISOLEUCINE

All 1-liter cultures of B. licheniformis were growing in early-log phase (about 1 g/l) when the radioactive substrate was added. [1-¹⁴C] Palmitate (0.625 μ moles, 30 μ c) was added to cultures growing at 35° (Expt 1) or at 20° (Expt 2) while [U-¹⁴C]-L-isoleucine (0.04 μ moles, 10 μ c) was added to a culture growing at 35° (Expt 3). All cells were harvested by centrifugation in late-log phase (6.2 - 6.7 g/l) and the cell pellets were extracted by the modified Folch procedure. The phospholipids were isolated as a group from each experiment as described for Fig. 9 and then divided into several portions. One portion from each experiment was assayed for desaturation by the mercuric acetate technique. A second portion from each was separated by thin-layer chromatography into 3 fractions (phosphatidyl glycerol, phosphatidyl ethanolamine and other [unidentified] phospholipids) as described for Fig. 9. The relative distribution of the radioactivity in the 1 and 2 positions of each isolated, identified, phospholipid was then determined by phospholipase A hydrolysis of the fatty acyl group at the 2-position, followed by thin-layer chromatography to separate the products, all as described in the methods section. For comparison purposes, a second group of experiments using B. megaterium 14581 (Expt 4-6) was carried out in the same manner as described for B. licheniformis. In addition, however, in Expt 5, the lipid fractions, separated after phospholipase A hydrolysis of phosphatidyl glycerol and phosphatidyl ethanolamine, were eluted from the thin-layer plates, transesterified with HCl-methanol and assayed for unsaturated fatty acids

by the mercuric acetate technique. These latter results, expressed as % desaturation found in each fraction, are shown in parentheses.

TABLE IV (Cont.)

Expt	Organism, temp., ¹⁴ C-substrate	% of ¹⁴ C- activity in unsaturated acids	Phosphatidyl glycerol (%)			Phosphatidyl ethanolamine (%)			Other phospho- lipids
			1	2	total	1	2	total	
			position	position		position	position		total
1	<u>B. licheniformis</u> 35° palmitate	48	14.7	24.0	38.7	37.4	18.4	55.8	5.5
2	<u>B. licheniformis</u> 20° palmitate	86	6.4	20.3	26.7	42.9	27.4	70.3	2.9
3	<u>B. licheniformis</u> 35° L-isoleucine	8.5	4.2	37.3	41.7	4.8	26.9	31.7	26.8
4	<u>B. megaterium</u> 35° palmitate	1.7	10.5	42.1	52.6	33.7	13.1	46.8	0.6
5	B. megaterium	60	6.3	56.7	63.0	14.9	19.0	33.9	3.1

TABLE IV (Cont.)

	20°		(52%)	(61%)	(57%)	(45%)	(70%)	(62%)	
	palmitate								
6	<u>B. megaterium</u>	0.7	5.3	61.1	66.4	4.2	21.9	26.1	7.5
	35°								
	L-isoleucine								

Chang & Faller

FIG 1

Chang + Fulco

FIG 4

Chang + fulcor

chang + fulv

% OF RECOVERED ACTIVITY

HOURS

Chang &
Fulco

1

2

RADIOACTIVITY

A

STANDARD

B

SOLVENT FRONT   METHYL ESTER   TRIGLYCERIDE   1,3-DIGLYCERIDE   1,2-DIGLYCERIDE   FREE FATTY ACID   MONOGLYCERIDE   ORIGIN

Chang &
Fulco

Fig 8