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THE BIOSYNTHESIS OF UNSATURATED FATTY ACIDS BY BACILLI

III. Uptake and Utilization of Exogenous Palmitate^{*}

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The Biosynthesis of Unsaturated Fatty Acids by Bacilli

SUMMARY

The factors affecting the uptake, utilization and desaturation of exogenous palmitic acid by Bacillus megaterium 14581 have been elucidated and the validity of the in vivo assay for determining the relative levels of the temperature-induced Δ^5 -desaturating enzyme system in this organism has been established. It was found that, in the presence of glucose, palmitic-1- ^{14}C acid added to the cultures of B. megaterium is taken up rapidly and quantitatively by the cells. However, most of the ^{14}C -label is excreted back into the medium within 15 min. as water-soluble products (CO_2 , acetate, etc.) presumably derived from β -oxidation of the added palmitate-1- ^{14}C . The remaining palmitate-1- ^{14}C (about 30% of the amount added) mixes with a small endogenous pool of palmitate (35 μM moles per g of cells) which is readily desaturated but is completely stable to β -oxidation. In the absence of glucose, β -oxidation of added palmitate-1- ^{14}C follows the same time pattern but a greater proportion of the label is excreted into the medium as oxidation products. Concomitantly, the endogenous pool of palmitate available for desaturation drops to zero and total desaturation is reduced to about 6-7% of that obtained in the presence of glucose. Considerable experimental evidence would indicate that neither palmityl CoA nor the palmitate of neutral or phospholipids are substrates for desaturation. Rather, it seems more probable that the true substrate for desaturation is the palmitate derived from fatty acid biosynthesis, presumably palmityl-ACP (the palmityl thioester of acyl carrier protein). Exogenously derived palmitate-1- ^{14}C is thought to enter this pool indirectly by first being incorporated into a small,

metabolically active pool of neutral and phospholipids and then exchanging rapidly with endogenous palmityl-ACP.

In the presence of glucose, the endogenous pool of palmitate available for desaturation was shown to remain constant during the period when Δ^5 -desaturating enzyme was induced from an undetectable level to maximal levels after transfer of a 35°-grown culture of B. megaterium to 20°. Furthermore, the pool size was shown to be independent of levels of substrate desaturation. In all cases, in the presence of glucose and chloramphenicol, the level of desaturation was determined solely by the level of Δ^5 -desaturating enzyme (i.e. was independent of exogenous substrate concentration, total pool size, pH, cell concentration, etc.) within wide experimental limits. This fact permitted the development of a precise and accurate assay procedure for the determination of relative desaturating enzyme levels and opened the way for the in vivo study of the temperature-mediated mechanisms which regulate unsaturated fatty acid biosynthesis in B. megaterium.

In previous papers of this series (1,2) it was shown that a number of *Bacillus* species and strains desaturate palmitic acid to cis-5-hexadecenoic acid in a reaction that is both oxygen and iron dependent and temperature regulated. Experiments with *Bacillus megaterium* ATCC 14581 demonstrated that the Δ^5 -desaturating enzyme was absent in cultures growing at 30-35° but was rapidly induced when a culture was transferred to 20°. This temperature-mediated induction was chloramphenicol sensitive but once the enzyme was induced, chloramphenicol did not block desaturation. In the course of this work, several additional temperature-mediated controls of palmitate desaturation have also been observed (3) and it was decided to study in detail the various ways in which temperature could regulate unsaturated fatty acid biosynthesis. It was first necessary however to determine what other factors could influence the utilization and desaturation of palmitate and to ascertain that the assay procedure, by taking these factors into account, would accurately reflect desaturating enzyme levels in any culture as a function of temperature. The experiments reported here accomplish these aims and in addition, provide some insight into several non-temperature-mediated processes which affect fatty acid utilization.

EXPERIMENTAL PROCEDURE

Growth and Incubation of Bacteria-*Bacillus megaterium* 14581 was obtained from the American Type Culture Collection and was grown on a salts-glucose-casamino acids medium as previously described (1,4). Cell concentrations were determined by optical density readings and are

expressed as g/liter packed wet weight of cells.

Substrates and Inhibitors—Palmitic-1-¹⁴C acid was purchased either from New England Nuclear Corporation or from Schwarz BioResearch, Inc. and was purified and utilized as described previously. Chloramphenicol, B grade, used as a protein synthesis inhibitor, was purchased from Calbiochem.

Assay for Desaturating Enzyme—Unless noted otherwise in the text, the assay procedure for Δ^5 -desaturating enzyme was carried out as follows: 5 ml of bacterial cell suspension (either in glucose-phosphate buffer or growth medium) were added to 5 ml of assay buffer containing potassium phosphate buffer, 0.05 M, pH 7.0, glucose, 10 g/liter and chloramphenicol 100 mg/liter. The temperature of the assay buffer was such that, upon mixing with the cell suspension, the final temperature of the mixture was $20 \pm 0.5^\circ \text{C}$. After mixing, palmitic-1-¹⁴C acid (2.5 μmoles in 10 μl ethanol) was added with a microliter syringe and the mixture was incubated at 20° with shaking for three hours. At the end of this period, the mixture was passed through a 0.8 μ MF-Millipore filter and the collected cells along with the filter were transferred to a 15 ml culture tube equipped with a Teflon-lined screwcap and containing 3 ml of 5% anhydrous HCl in methanol, 0.15 ml of 2,2-dimethoxypropane (99 + %, Aldrich Chemical Co., Inc.) and 1 mg each of palmitic and oleic acids as carriers. The tubes were closed, heated at $80\text{--}85^\circ$ overnight and then the methanolysis products extracted with 3, 5 ml volumes of n-pentane. The methyl esters, in pentane, were purified on silica gel, separated into saturated and unsaturated fractions by the formation of the mercuric acetate adducts and

silica gel chromatography and finally counted, all as described previously (1,4).

Counting Procedures—Radioactivity determinations were carried out on a Packard Tri-Carb liquid scintillation counter as previously described (1). Methyl esters and other liquid samples, including the mercuric acetate adducts were counted in a toluene solution of Permablend I (5.5 g/liter of toluene) purchased from the Packard Instrument Company, Inc., while aqueous samples, bacterial cells, and other non-lipid substances were counted in 10-15 ml of Scintisol Complete, purchased from Isolab, Inc. The MF-Millipore filters used to collect bacteria were soluble in both scintillator solutions.

RESULTS

Uptake and Distribution of Label from Added Palmitate-1-¹⁴C—From the results of experiments reported previously (1,4) it was apparent that when B. megaterium was incubated in air with palmitate-1-¹⁴C for several hours, in either growth medium or in buffer, much of the radioactivity originally added to the medium was not recovered in the harvested cells. If the cells were incubated anaerobically with palmitate-1-¹⁴C for 10 hours, as much as 85% of the added ¹⁴C activity could be recovered in the cells after harvesting. However, once air was admitted, this activity was rapidly lost so that, after an additional 10 hours of incubation in air, the recovery of activity dropped to between 7 and 21% (4). From these results it seemed likely that, under aerobic conditions, a major portion of the added palmitate-1-¹⁴C was being oxidized and the ¹⁴C activity was either being lost

as CO_2 or excreted into the medium as water-soluble products. The results of an experiment designed to test this hypothesis are shown in Figure 1.

← FIGURE 1

Thirty seconds after the addition of palmitic-1- ^{14}C to the culture medium almost 90% of the radioactivity is recovered in the harvested cells but this value dropped to 50% after 5 min. and to 35% after 20 min. After 20 min. the proportions of ^{14}C activity in the cells and in the culture medium remained relatively constant. When the filtrates were acidified to pH 2 with 6N HCl, about 40% of the radioactivity was lost, presumably as CO_2 . When the acidified filtrates were extracted with equal volumes of either n-pentane or chloroform (procedures which readily extract free fatty acids) less than 1% of the remaining radioactivity was found in the organic phase. However, 5 extractions of the acidified filtrate with equal volumes of diethyl ether removed about 70% of the residual activity. From these results, it was concluded that none of the ^{14}C activity present in the filtered medium was due to unmetabolized palmitic-1- ^{14}C acid. It is more likely that the filtrate radioactivity results from CO_2 and such water and ether soluble components as acetate, acetoacetate, and β -hydroxybutyrate derived from β -oxidation of the substrate. On the other hand, when the harvested cells were treated with HCl-methanol, almost all of the radioactivity was readily extractable with n-pentane and could be eluted from silica gel with 2% ether in n-pentane, a behavior typical of unsubstituted fatty methyl esters (in this case palmitate or its unsaturated derivative).

Effects of Glucose and pH on Incorporation and Desaturation of Palmitic-1-¹⁴C Acid—It was noted in a number of experiments that when cells were resuspended in either glucose-phosphate buffer or in fresh growth medium, the recovery of radioactivity from added palmitic-1-¹⁴C acid was higher in these cells than in cells harvested after incubation with palmitic-1-¹⁴C acid in "aged" medium (i.e. medium obtained by centrifugation of stationary-phase cultures. This was true both in the presence or absence of chloramphenicol. When cells were resuspended in a mixture of fresh and aged medium (1:4) there was a two-fold increase in radioactivity recovered in the harvested cells after a 2-hour incubation with palmitic-1-¹⁴C acid, with a concomitant drop of radioactivity excreted into the medium. This suggested that fresh medium was either inhibiting the β -oxidation of the added palmitic-1-¹⁴C acid or stimulating the incorporation of palmitate into a fraction not available for β -oxidation. Since "aged" medium had little or no glucose remaining and, in addition, had a lower pH than fresh medium, the possible effects of glucose and pH on recovery of radioactivity were examined.

Figure 2 shows how these factors affect the recovery of radioactivity in the harvested cells. It can be seen that pH changes do not affect the recovery of ¹⁴C activity in cells incubated in the absence of glucose and have only a slight effect on cells incubated with glucose. The effect of glucose however is quite dramatic. At all pH's there is at least a 2-fold increase in recovery of radioactive fatty acids when glucose is included in the incubation mixture. More important, however, the addition of glucose also stimulates desaturation as illustrated in Figure 3.

← FIGURE 2

← FIGURE 3

In the presence of glucose (curve A), desaturation remains relatively constant at about 42-45% over the pH range used while in the absence of glucose (curve B) desaturation drops from 27% at pH 6.4 to about 18% at pH 7.4.

The Effects of Changes in Cell and Glucose Concentrations on Incorporation and Desaturation of Palmitic-1-¹⁴C Acid—In order to determine how variations in glucose levels affected palmitate uptake and desaturation, a series of assay incubations (i.e. 20°, 3 hours, in the presence of chloramphenicol), with palmitic-1-¹⁴C acid were carried out either in the absence of glucose or at glucose concentrations ranging from 1.25-10.0 g/liter. In addition, the cell concentrations were varied from 1.25 to 10.0 g/liter at each glucose level. As will be shown, the results from this experiment not only demonstrate how glucose is acting to affect palmitate pool size and desaturation, but they also permit the calculation of absolute pool sizes of palmitate and demonstrate that the amount of palmitate desaturated is relatively independent of pool size in either the presence or absence of glucose. These results are shown in Table I and in Figure 4. It can be seen (Table I) that varying the glucose concentrations from 1.25 to 10.0 g/liter has no significant effect on either the uptake or percent desaturation of radioactive palmitate by the cells although, in the complete absence of glucose, both palmitate-1-¹⁴C incorporation and desaturation are reduced. At constant glucose concentration, the lowering of cell concentration from 10 to 1.25 g/liter slightly reduces the total incorporation of palmitate-1-¹⁴C. There is a much greater reduction, however, on palmitate-1-¹⁴C desaturation (about

TABLE I
FIGURE 4

2-fold). This latter fact suggests that, at low cell concentrations, added palmitate-1-¹⁴C is not acting as a tracer but is significantly increasing the pool size of palmitate available for desaturation. This follows from the fact that, at a constant glucose concentration, the absolute amount of unsaturated fatty acid formed per gram of cells must be a constant. If the radioactive palmitate was incorporated only as a tracer (i.e. did not significantly increase the endogenous pool size of palmitate available for desaturation), the percent desaturation of palmitate-1-¹⁴C should also be a constant. A decrease in percent desaturation with increasing palmitate incorporation per gram of cells indicates an increasing total pool size (palmitate-1-¹⁴C incorporated + endogenous available palmitate). The endogenous pool of palmitate available for desaturation can be readily calculated by solving for 2 simultaneous equations of the form:

$$(D_A) (PI_A + PE) = K = (D_B) (PI_B + PE) \quad (1)$$

where D = the fraction of radioactive palmitate desaturated, PI = the μ moles of palmitate-1-¹⁴C incorporated per g cells, PE = the μ moles of palmitate per g cells in the endogenous pool available for desaturation and K = the μ moles of palmitate desaturated per g cells and is assumed to be a constant. This equation can be applied to any set of two determinations (A and B) to find PE by rearranging as follows:

$$PE = \frac{D_A PI_A - D_B PI_B}{D_B - D_A} \quad (2)$$

When these calculations are carried out, a relatively constant pool size of about 35 μ moles of available endogenous palmitate per g cells¹ is obtained for all cell densities at glucose concentrations ranging from 1.25-10.0 g/liter. On the other hand, in the complete absence of glucose, Equation 2 indicates that the endogenous palmitate pool (PE) available for desaturation is now zero and, in addition, both palmitate-1-¹⁴C incorporation (PI) and the fraction desaturated (D) have been drastically reduced (see Table I). The total μ moles of palmitate-1-¹⁴C desaturated per g cells (K) can now be calculated for each sample by the equation,

$$K = (D) (PI + PE) \quad (3)$$

since D and PI are experimental values and PE was calculated from Equation 2. This was done using both PE = 35 μ moles and PE = 0 for all samples (Table I, last 2 columns). It can be seen that, for those incubations carried out in the presence of glucose, K is a constant when PE = 35 μ moles while, in the absence of glucose, K is constant when PE = 0. These relationships are more clearly seen in Figure 4 where the values for K calculated for pool size of 0 and 35 μ moles respectively, are plotted against cell concentrations for both those samples containing glucose (K values averaged) and those without glucose. These curves, coupled with the data in Table I imply that all of the palmitate-1-¹⁴C incorporated has mixed with the endogenous pool of palmitate available for desaturation (but see Footnote 1) and that the amount of unsaturated fatty acid formed per g of cells is constant and independent of total

pool size (PI + PE) and of glucose levels down to 1.25 g/liter. In the complete absence of glucose, there is no endogenous pool of palmitate available for desaturation and furthermore, the absolute level of desaturation (utilizing palmitate-1-¹⁴C taken up from the medium) is reduced about 16 fold relative to the glucose-containing samples. Even in the absence of glucose, however, desaturation is independent of the total pool size (which in this case is simply PI, since PE = 0).

The Effects of Increasing Levels of Added Palmitic-1-¹⁴C Acid on Incorporation and Desaturation—In the next experiment, both cell concentrations and the amounts of palmitate-1-¹⁴C added to the incubation medium were varied in the presence of a constant amount of glucose and each sample was assayed in the usual way for desaturation. The results of this experiment are illustrated in Table II. It can be seen that at high cell concentrations and/or low levels of palmitate incorporation, there is relatively little variation in percent desaturation. In these instances, the amounts of palmitate-1-¹⁴C incorporated are small compared to the endogenous pool available for desaturation. At low cell concentrations or at high palmitate-1-¹⁴C incorporation levels, this is no longer true and the size of the endogenous pool must be considered before the data can be used to calculate desaturation enzyme activities. The endogenous pool size (PE) can be calculated by Equation 2 and when this is done for each pair of values, an average pool size of 35 μ moles of palmitate per g of cells is obtained, a value in agreement with that obtained in the previous experiment. When the total palmitate desaturated per g of cells (K) is calculated using a value of PE = 35 in Equation 3

← TABLE II

an average K value of 21.2 μ moles is obtained for all samples. The K values for individual samples (last column, Table II) do not deviate significantly from this figure. Thus, the results of this experiment confirm those obtained in the previous experiments and demonstrate that the absolute amount of palmitate synthesized is independent of the total pool size of palmitate available for desaturation. The data also shows that the total amount of palmitate-1-¹⁴C incorporated is a function both of the amount of palmitate added to the medium and of cell concentration. However, as Figure 5 illustrates, these relationships are not simple ones and no equation has yet been derived to determine incorporation from given cell and substrate concentrations. Undoubtedly, the situation is complicated by the action of the competing systems involved in the β -oxidation of the added palmitate-1-¹⁴C.

← FIGURE 5

Variation of Palmitate Pool Size During Desaturase Induction—Once it was shown that the absolute level of desaturation was independent of palmitate pool size it now became possible to experimentally determine whether the level of endogenous palmitate available for desaturation varied during the course of temperature-induced synthesis of the Δ^5 -desaturating enzyme. By assaying for desaturation during the course of induction and utilizing either 2 different levels of added palmitic-1-¹⁴C acid or 2 different cell concentrations or both at each time point, the data could be substituted into Equation 2 to obtain a value for PE at each point. Although the precision of such a method is poor (since small errors in the determination of percent desaturation can become very large errors in the determination of PE because of the nature of the equation), any large changes in PE with

induction time should be detectable. Table III shows the results of 2 such experiments. Although there is considerable scatter in the values obtained for PE, these values show no obvious trend towards increasing or decreasing pool size during the course of induction. Indeed, considering the limited precision of the method, the values for PE are reasonably constant. These results are reassuring in that it allows one to utilize percent desaturation of added palmitic-1-¹⁴C acid (within certain limits - see following experiment) as a measure of absolute desaturase activity.

TABLE III

The Effect of Incubation Time on the Assay for Desaturating Enzyme

The in vivo assay for desaturating enzyme used for the experiments reported here is described in the methods section (see Experimental Procedure) and consists of measuring the percentage of added palmitic-1-¹⁴C desaturated by the cells in 3 hours under standard conditions. This procedure was adopted because the desaturating enzyme is unstable in the assay buffer (which contains chloramphenicol to block further enzyme synthesis) and undergoes irreversible inactivation which is rapid relative to the rate of desaturation. The inactivation follows first order kinetics at a rate that is temperature dependent (see the following paper in this series). Thus, because of these factors as well as the short but significant time required for the uptake of the added substrate and the relatively large experimental errors involved in measuring low levels of desaturation at short times, the initial reaction rates could not be measured with any accuracy². Instead, a 3-hour assay incubation was chosen since, at the end of this period, desaturation activity was

no longer measurable because of rapid enzyme inactivation and thus, in effect, each desaturation assay was carried to completion. As long as the rate of desaturation during the assay period was determined only by enzyme concentration, which was decreasing in a first-order fashion, the actual time of incubation used in the assay should have no significant effect on the relative amounts of enzyme measured. In other words, if one sample (A), initially contains twice as much desaturating enzyme as another (B), this relationship should hold throughout the assay period³ and the ratio of desaturating activity of A to that of B would be 2, regardless of the time period of assay. That this is indeed true is demonstrated by the results of an experiment in which increasing levels of Δ^5 -desaturating enzyme were induced and then each level was measured at 5 different assay times (15 to 180 min.). As the results show (Table IV), with the exception of the 15 min. assay periods, the relative values obtained for the various enzyme levels are independent of assay times⁴. Similar experiments have shown that, as long as no more than about 55% of the added palmitate is desaturated in a 3-hour assay period, the correlation between percent desaturation and true enzyme level holds. However, when initial enzyme levels are so high that much more than about 50% of the added palmitate is desaturated in the 3-hour assay, the correlation no longer holds and a shorter assay time must be chosen such that, at termination, no more than 50-55% of the added palmitate is desaturated. The validity of the assay system is further confirmed by the finding that half life determinations were independent of assay times ranging from 30 to 180 min. (see the following paper in this series).

TABLE IV

DISCUSSION

The major purpose of the experiments described in this paper was to lay the groundwork for the detailed investigation, in vivo of the mechanisms by which temperature changes affect the biosynthesis of unsaturated fatty acids in Bacillus megaterium. In so doing, the action of several non-temperature regulated factors which can affect unsaturated fatty biosynthesis were elucidated and the validity of the assay system used to determine desaturating enzyme levels was established.

The results shown in Figure 1 and reported in the text show that most of the exogenously supplied palmitic-1-¹⁴C acid is degraded to CO₂ and other water-soluble products within minutes after its uptake by the bacterial cells, presumably by a very active β -oxidation system⁵. However, after the first 15 min. the incorporated palmitate is no longer subject to β -oxidation but has become part of a small pool (approximately 35 μ moles per g cells) of palmitate that is really available for desaturation. The remarkable stability to oxidation of the incorporated palmitic-1-¹⁴C after the first 15 min. of incubation is further demonstrated by the data in Table IV which shows that there is virtually no oxidation of palmitate-1-¹⁴C (and its desaturation product) in samples taken from 15 min. to 3 hours. Yet, obviously, the stable pool containing palmitate-1-¹⁴C is readily available to the desaturating system throughout this period. Since the β -oxidation system remains active throughout the duration of the assay⁶, two other possibilities remain that might explain the observations: either the β -oxidation system is located outside or within the cell membrane and palmitate-1-¹⁴C is available

as a substrate for oxidation only during passage through the membrane, or else the pool of palmitate which is not susceptible to oxidation is present in forms not utilizable by the β -oxidation enzymes (i.e. not present as or readily convertible to palmityl CoA)⁵. At the present time, work in this laboratory which will be published at a later date would favor the latter explanation. We now feel that the true substrate for desaturation in Bacilli is most probably the acyl carrier protein (ACP) derivative and we are presently engaged in preparing palmityl-1-¹⁴C-ACP for testing in cell free systems.

The finding that glucose is necessary for maximal desaturation activity and for the maintenance of the palmitate pool available for desaturation (PE) supports the view that the endogenous substrate for desaturation is not palmityl-CoA but some other derivative. Obviously, the short-term absence of glucose does not detectably depress the activation of palmitic acid to the CoA ester since an even greater proportion of the added substrate is diverted to β -oxidation under these conditions. Yet total desaturation is reduced to less than 7% of the level found in the presence of glucose and the endogenous pool of palmitate (PE) is reduced to zero.

In the absence of glucose, fatty acid synthesis should be strongly inhibited or abolished (5) and in the light of the work by Butterworth and Bloch (6) that conclusively demonstrates the involvement of ACP in the fatty acid synthesizing system of Bacilli, it is not unlikely that the endogenous pool of palmitate available for desaturation is actually palmityl-ACP derived from fatty acid biosynthesis. Nevertheless, if

palmityl-ACP is the true substrate for desaturation, a mechanism must exist for the conversion of exogenous palmitic-1-¹⁴C acid to the ACP derivative since that which survives β -oxidation rapidly becomes part of this pool. We feel that palmitate is not activated directly to the ACP derivative but rather enters the pool in an exchange reaction similar to that described by Ailhaud, Vagelos, and Goldfine (7). Norman Chang⁷, in this laboratory, has shown that exogenous palmitic-1-¹⁴C acid is rapidly incorporated into neutral lipids and phospholipids⁸ of B. megaterium, primarily in the 2-position of the glycerol moiety but that these intact lipids are not themselves substrates for desaturation. Rather, there seems to be a rapid exchange of label between these lipids and the true substrate for desaturation, presumably palmityl-ACP. However, the question seems to be an exceedingly complex one and further speculation is not justified on the evidence available to us at the present time.

The results of the remaining experiments are self-explanatory and require little discussion. These experiments were designed to establish the validity of the in vivo assay procedure for desaturating enzyme and to define the limits within which the assay system could be used. The fact that, in the presence of glucose and chloramphenicol, desaturation is enzyme limited and the endogenous pool of palmitate available for desaturation is relatively constant throughout the period of temperature-mediated induction allows us to utilize percent desaturation as an accurate measure of relative levels of desaturating enzyme. When the amount of added palmitic-1-¹⁴C acid is small compared to the endogenous pool available for desaturation and the percent desaturation does not exceed certain

limits within the assay period, then percent desaturation values may be equated to "enzyme units" within the same experiment. The fortuitous fact that, in the presence of chloramphenicol, desaturase activity decays with strict first-order kinetics eliminates the necessity for measuring initial reaction rates and contributes to the precision of the assay by essentially allowing the desaturation reaction to go to completion over a relatively long time period. The usefulness of this procedure in the study of temperature-mediated controls in desaturation is apparent in the following paper in this series.

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FOOTNOTES

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1. One must distinguish here between the endogenous pool size of palmitate available for desaturation, PE, which is about 35 μ moles of palmitate per g of cells and the total palmitate per g of cells which is much larger (800 μ moles) but most of which is not available for desaturation and does not mix with added palmitate-1- 14 C. The total palmitate was determined by gas chromatography of the methyl esters obtained from HCl-methanol treatment of the cells.
 2. For example, even if the desaturating enzyme were relatively stable so that corrections for first-order loss of activity could be ignored, variations in the rate of palmitic-1- 14 C acid uptake and filtering time at the termination of the assay would have to be

considered. Even if the effect of these factors could be determined, the percent desaturation values at short times (even for high enzyme levels) would be so low that the limitations in precision of the mercuric acetate assay would make acceptable accuracy difficult to obtain.

3. Assuming, of course, that there is no significant deviation from a first-order rate of enzyme decay. This point is thoroughly discussed in the following paper of this series.
4. The absolute error due to the finite time required for the uptake of substrate should, of course, decrease with assay time, but not in a linear fashion. The chief source of error at short assay times however, can probably be ascribed to the lack of precision of the mercuric acetate method for determining percent desaturation. The background varies between 0.6-1.1% of the total incorporated counts with an average background (blank value) of 0.8%. This value is subtracted from all determinations.
5. This system has been examined in vitro in B. megaterium (G. Oliver and A. Fulco, unpublished results) and as expected, palmityl CoA but not free palmitate is utilized as a substrate for β -oxidation.
6. See the following paper in this series.
7. N. Chang, Ph.D. Thesis, UCLA, August, 1971.

8. This seems to be a small, metabolically active pool of lipids and is quite distinct from a much larger pool (presumably present in previously synthesized cell membranes) which is relatively inert metabolically and does not become labeled. The palmitate of this large pool, as shown by gas chromatography, is not significantly desaturated, at least during short-term (6 hours or less) incubations at 20°.

LEGENDS FOR FIGURES

Fig. 1. Distribution of palmitate-1-¹⁴C during a 2-hour incubation with B. megaterium 14581. B. megaterium 14581 (200 ml.) was grown at 33° to a density of 9.9 g/liter (late log phase) and then cooled rapidly in ice to 20° and transferred to the 20° shaker. Palmitic-1-¹⁴C (50 μ moles, 4.3×10^6 cpm) was immediately added and duplicate 5 ml. samples were withdrawn at intervals and pipeted on millipore filters. The collected cells and the filter from one duplicate were counted directly in 15 ml. of Scintisol Complete while the second cells-filter sample was subjected to methanolysis and subsequent pentane extraction (see text). The filtrates from the duplicate samples were combined, a portion was counted directly in Scintisol Complete while the remainder was treated as described in the text. In Figure 1, curve A shows the recovery of counts from the cells obtained from 5 ml. of medium while curve B shows the radioactivity recovered in 5 ml of filtrate. Total activity recovered averaged 95.0% of that added as palmitate-1-¹⁴C with a range of recovery between 90.6 and 100.5%.

Fig. 2. The effects of glucose and pH on the incorporation of palmitic-1-¹⁴C acid. B. megaterium 14581 was grown to late log phase (10.3 g/liter) at 35°, cooled rapidly in ice to 20° and then incubated at 20° for 1 hour (to a final density of 11.6 g/liter). The cells were packed by centrifugation at 20° for 5 min. at

10,000 x g and resuspended in distilled water to a concentration of 25 g/liter. Two ml. of this cell suspension were added to each of a series of flasks, each containing 1 mg. of chloramphenicol, 2 ml. of potassium phosphate buffer (0.2 M) of the appropriate pH, and either 6 ml. of glucose solution (50 mg. glucose) or 6 ml. of distilled water to give a final volume of 10 ml. After the addition of cells, 10 μ moles of palmitic- $1-^{14}\text{C}$ acid (300,000 cpm) were added and the flasks were incubated at 20° for 3 hours. The cells were then harvested by millipore filtration, subjected to methanolysis and the resulting methyl esters were purified on silica gel. Curve A (Figure 2) shows the counts obtained in the purified methyl esters from the glucose-incubated cells while curve B shows the methyl ester counts in the absence of glucose.

Fig. 3. The effects of glucose and pH on the desaturation of palmitate- $1-^{14}\text{C}$. The purified methyl ester samples obtained from the experiment described under Figure 2 were separated into saturated and unsaturated fractions and counted. Curves A and B (Figure 3) show the percent desaturation of each sample based on the recovery of radioactivity in the saturated and unsaturated fractions. Curve A, glucose-incubated cells; curve B, no glucose.

Fig. 4. Effect of increasing cell concentration on palmitate desaturation. For the experiment described in Table I, the total amount of palmitate desaturated per g of cells was calculated for each

sample by multiplying percent desaturation by the total pool size of palmitate available for desaturation as given by Equation 3 in the text. Two calculations were carried out for each sample using endogenous pool size (PE) of 35 and 0 μ moles palmitate per g cells respectively (see Table I). The curves designated "+G" are for those samples assayed in the presence of glucose and each point represents an average of the values obtained at glucose concentrations ranging from 1.25 to 10.0 g/liter. The curves marked "-G" are for samples assayed in the absence of glucose.

Fig. 5. Incorporation of palmitic-1-¹⁴C acid as a function of cell concentration and amount of substrate added. The data from which these curves are derived are shown in Table II. Each curve is identified by a number which corresponds to the μ moles of palmitic-1-¹⁴C acid added to the 10 ml. incubation mixture.

TABLE I

The effects of changes in glucose and cell concentrations on the uptake and desaturation of palmitate-1-¹⁴C

B. megaterium 14581 was grown at 34° to late log phase (9.3 g/liter), cooled rapidly to 20° and then incubated at 20° for 75 min to a final density of 11.0 g/liter. The cells were harvested by centrifugation and resuspended in distilled water at 20° at concentrations ranging from 100 to 12.5 mg/ml. Cells (1 ml of the appropriate concentration) were then added to a series of flasks, each containing 1 mg of chloramphenicol, 2 ml of 0.2 M potassium phosphate buffer, pH 7.0, and either distilled water or glucose solutions to give a final volume of 10 ml. Palmitic-1-¹⁴C acid (10 μ moles) was then added and incubation and assays carried out as described under Figures 2 and 3.

Cells	Glucose	Palmitate-1- ¹⁴ C incorporated per g cells (PI)	Palmitate- 1- ¹⁴ C desaturated (D) (100)	Total palmitate desaturated per g cells (K)	
				Endogenous pool size (PE) = 0	Endogenous pool size (PE) = 35 μ moles/g
g/liter	g/liter	μ moles	%	μ moles	
10	0	3.8	36.7	1.39	14.74
	1.25	7.0	52.3	3.67	21.97
	2.50	8.2	51.4	4.24	22.23
	5.00	7.7	54.9	4.21	23.42
	10.00	8.2	57.7	4.73	24.92

5	0	4.8	32.8	1.59	13.06
	1.25	14.0	47.6	6.65	23.31
	2.50	13.5	49.1	6.63	23.81
	5.00	13.9	47.0	6.52	22.97
	10.00	14.6	44.0	6.40	21.80
2.5	0	10.4	14.0	1.45	6.35
	1.25	22.1	42.7	9.42	24.37
	2.50	27.7	39.4	10.90	24.70
	5.00	22.1	42.6	9.42	24.33
	10.00	24.8	41.0	10.18	24.53
1.25	0	14.9	9.5	1.41	4.74
	1.25	44.8	29.1	13.04	23.23
	2.50	42.8	30.0	12.85	23.35
	5.00	53.8	26.2	14.10	23.27
	10.00	40.2	29.0	11.67	21.82

TABLE II

The effects of increasing amounts of added palmitic-1-¹⁴C acid on incorporation and desaturation

B. megaterium 14581 was grown at 34° to late log phase (8.1 g/liter), cooled rapidly to 20° in ice, and then incubated at 20° for 70 min to a final density of 9.4 g/liter. The cells were harvested and resuspended in distilled water and added to flasks containing the chloramphenicol-glucose-phosphate buffer assay mixture (as described in Table I) to give final cell concentrations ranging from 0.625 to 10.0 g/liter in a final volume of 10 ml. Palmitic-1-¹⁴C acid (20 μ c/ μ mole) was added to each sample in concentrations ranging from 1 to 25 μ moles per 10 ml. Incubation and assays were carried out as described for the experiment illustrated under Figures 2 and 3.

Cells	Palmitate-1- ¹⁴ C added to the medium	Palmitate-1- ¹⁴ C incorporated per g cells (PI)	Palmitate-1- ¹⁴ C desaturated (D) (100)	Total palmitate desaturated per g cells (K) PE = 35
g/liter	μ moles	μ moles	%	μ moles
10	1.0	2.7	57.1	21.5
	2.5	6.0	51.6	21.2
	5.0	8.2	51.8	22.4

	10	13.0	49.8	23.9
	25	a	a	a
5	1.0	4.7	49.1	19.5
	2.5	10.4	47.3	21.5
	5.0	13.1	44.9	21.6
	10	19.7	41.3	22.6
	25	33.0	33.3	22.6
2.5	1.0	6.4	50.8	21.0
	2.5	15.2	37.0	18.6
	5.0	20.7	31.6	17.6
	10	30.9	29.7	19.6
	25	63.0	25.2	24.7
0.625	1.0	21.0	43.5	24.4
	2.5	47.7	22.2	18.4
	5.0	57.9	a	a
	10	108.1	12.1	17.3
	25	286.9	6.7	21.6

^aSample lost

TABLE III

Variation of palmitate endogenous pool size during the course of temperature-induction of desaturating enzyme

Experiment 1: B. megaterium 14581 was grown at 35° to late log phase (9.3 g/liter), cooled quickly in ice to 20° to initiate induction of the desaturating enzyme and then incubated at 20° for 5 hours. During this period, cell density increased to 13.0 g/liter. At various time intervals after the start of the 20° incubation, 5 ml samples were withdrawn and added to 5 ml of assay buffer (see Table I) and desaturation activity was determined by the usual procedure in the presence of either (A), 2.5 μ M moles or (B), 10 μ M moles of palmitic-1-¹⁴C acid added to the final 10 ml assay mixture.

Experiment 2: B. megaterium 14581 was grown at 33° to late log phase (10.3 g/liter), cooled to 20° in ice and then incubated at 20° for 2-1/2 hours to a final cell density of 15.2 g/liter. For part (A), 5 ml of cell suspension was pipetted into 5 ml of assay buffer at various time intervals and desaturation activity determined in the presence of 1 μ M moles of palmitic-1-¹⁴C acid. For part (B), 2.5 ml of cell suspension were pipetted into a mixture of 5 ml of assay buffer and 2.5 ml of "aged" medium^a and assayed for desaturation activity in the presence of 8 μ M moles of palmitic-1-¹⁴C acid in the usual manner.

Experiment number	Time after transfer to 20°	Palmitate-1- ¹⁴ C incorporated per gram of cells (PI)		Palmitate-1- ¹⁴ C desaturated (D) (100)		Endogenous palmitate pool size (PE)
		A	B	A	B	
	min	μ M moles		%		μ M moles/g cells

1	20	5.69	10.96	21.2	18.7	33.7
	30	3.90	9.05	25.1	23.0	52.6
	45	3.02	7.66	33.7	31.3	57.5
	60	3.08	8.09	b	34.0	b
	90	3.68	10.07	45.7	38.9	32.9
	120	3.70	10.90	61.0	55.7	71.9
	180	5.02	12.86	68.3	62.4	77.9
	240	4.00	11.82	72.0	62.0	44.5
	300	5.14	10.28	73.0	62.6	25.8
2	20	2.93	13.89	20.2	14.3	23.6
	30	3.38	13.99	27.2	22.7	50.2
	45	2.34	15.39	38.7	33.8	87.7
	60	2.55	16.59	48.6	38.3	49.7
	75	3.60	17.74	57.7	46.5	55.1
	90	2.93	18.97	61.8	50.1	65.9
	120	2.84	16.73	65.4	48.8	33.0
	150	3.39	18.01	64.8	54.3	72.2

^a"Aged" medium was prepared by centrifuging a portion of the 35° culture during the late log phase and decanting the supernatant solution for use. This was done to make the A and B assay conditions as identical as possible except, of course, for cell and substrate concentrations.

^bSample was lost before % desaturation could be determined.

TABLE IV

The effect of incubation time on the assay for desaturating enzyme

B. megaterium 14581 was grown at 35° to late log phase (11.7 g/liter), cooled rapidly in ice to 20° to initiate induction of the desaturating enzyme and then transferred to 20° for continued incubation. At 15 min time intervals, 50 ml samples were withdrawn and mixed with 50 ml of the usual chloramphenicol-glucose-phosphate assay buffer. To the final 100 ml assay volume was added 25 μ moles of palmitic-1-¹⁴C acid, shaking was begun and 10 ml aliquots of the assay mixtures were withdrawn at time intervals ranging from 15 to 180 min and percent desaturation determined in the usual way except for the variations of the assay period from 15 min to the usual 180 min.

Enzyme induction period ^a	Assay period ^b	Palmitate-1- ¹⁴ C incorporated per g cells (PI)	Palmitate-1- ¹⁴ C desaturated	Desaturating enzyme level ^c
min	min	μ moles	%	
15	15	4.36	0.29	0.04
	30	4.03	0.51	0.03
	45	4.33	1.28	0.06
	60	4.41	1.43	0.06
	180	4.24	3.08	0.11

30	15	3.48	2.09	0.29
	30	3.34	6.36	0.41
	45	3.60	8.96	0.39
	60	3.64	10.32	0.44
	180	3.49	12.90	0.46
45	15	2.92	3.26	0.44
	30	2.56	10.61	0.69
	45	2.93	16.43	0.71
	60	2.73	19.05	0.81
	180	2.55	23.80	0.84
60	15	3.08	7.31	1.00
	30	2.67	15.33	1.00
	45	2.53	23.14	1.00
	60	2.79	23.50	1.00
	180	2.66	28.25	1.00

^aEquivalent to time of incubation at 20° before sample was taken for assay.

^bTerminated by millipore filtration of cells.

^cThe enzyme level after 60 min induction was arbitrarily assigned a value of 1.00. The relative levels at induction times of 45, 30 and 15 min at various assay times were then determined by dividing the percent desaturation at any given induction period and assay time by the percent desaturation obtained for the 60 min induction at the same assay time.

(Equation 2 - duplicate:)

$$PE = \frac{D_A PI_A - D_B PI_B}{D_B - D_A}$$

mM MOLES PALMITATE DESATURATED PER G. CELLS

mμ MOLES PALMITATE-1-C¹⁴ INCORPORATED

CELL CONCENTRATION (g/liter)

0.625

2.5

5.0

10.0

1.0

2.5

5

10

25