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THE BIOSYNTHESIS OF UNSATURATED FATTY ACIDS BY BACILLI

IV. Temperature-Mediated Control Mechanisms*

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The Biosynthesis of Unsaturated Fatty Acids by Bacilli

SUMMARY

Three control-mechanisms have been demonstrated in Bacillus megaterium which regulate the level of Δ^5 -desaturating enzyme, and hence the rate of unsaturated fatty acid biosynthesis, in response to temperature changes in the growth or incubation medium. One control process mediated by temperature is that of desaturase induction. A culture, growing at 35° does not synthesize unsaturated fatty acids. When the culture is transferred to 20° however, the synthesis of desaturase begins and continues at an accelerating rate for at least one hour. The induction process is blocked by chloramphenicol and the evidence suggests that both the desaturase synthesizing system and the desaturase itself are absent in cultures growing at 35° and must be induced at 20° by a process requiring protein synthesis.

A second control process responsive to temperature is the irreversible inactivation of the desaturating enzyme. The inactivation process, in vivo, follows strict first-order kinetics at all temperatures and the enzyme half-life is determined solely by the incubation temperature. The rate of inactivation of the desaturase is extremely sensitive to slight changes in temperature. At temperatures near 20° a decrease of less than 2° in the temperature of the incubation medium results in a more than 2-fold increase in the half-life of the desaturating enzyme. Temperature-mediated irreversible inactivation is probably the most important mechanism for regulating the steady state level of desaturase during the exponential phase of cell growth at temperatures near 20°.

A third process, the decay of the desaturase synthesizing system, is observed when a culture that has undergone induction at 20° is transferred

back to 34°. Not only is the irreversible first-order inactivation of the desaturating enzyme accelerated after transfer, but the desaturase synthesizing system itself immediately begins to decay at a rate which follows first-order kinetics. The kinetics of decay as well as several other characteristics of the process are consistent with (but do not conclusively demonstrate) a mechanism involving the temperature-triggered synthesis of a repressor for the desaturase synthesis system.

There is also some evidence for a rapid, temperature-reversible desaturation of desaturase at 34° which is superimposed on the slower, irreversible first-order inactivation of the desaturating enzyme.

Finally, equations are derived which enable one to calculate, as a function of time, the individual contribution of each of these temperature-dependent processes in regulating the level of desaturating enzyme at a given temperature in cultures of B. megaterium.

The influence of temperature on the unsaturated fatty acid composition of many organisms is well established, with the usual observation being that a decrease in environmental temperature favors a relative increase in unsaturated fatty acids at the expense of their saturated analogs. There is now considerable evidence that this phenomenon is an adaptive process on the part of the organism. Apparently the inverse relationship between temperature and unsaturated fatty acid composition serves to supply the optimal mixture of saturated and unsaturated lipids for cell membrane synthesis to maintain membrane integrity and function in the face of changing environmental temperature (1-8).

There are basically two ways by which temperature could act to regulate the proportion of unsaturated fatty acids in membranes. One way would involve a temperature effect on the enzymes which incorporate fatty acids into the membrane lipids. If, for example, temperature changes altered the specificity of selection (i.e., a lower temperature favoring a relative increase in the incorporation of unsaturated fatty acids into specific membrane lipids) then temperature adaptation could take place without requiring an overall change in unsaturated fatty acid biosynthesis. Indeed, just such a mechanism has recently been reported by Sinensky (9) who showed that the proportion of oleate to stearate incorporated from the medium into E. coli phospholipids increased with decreasing temperature. In E. coli extracts, the relative rates of transacylation of palmityl CoA and oleyl CoA were shown to vary as a function of temperature on the relative activity of a transacylase rather than on enzyme synthesis.

A second mechanism for the temperature controlled regulation of membrane fatty acid composition would be a direct effect on the relative amounts of saturated and unsaturated fatty acids produced, so that, at lower temperatures, more unsaturated fatty acids would be made available for incorporation into membrane lipids. In this case, the incorporation of the fatty acids need not be temperature-regulated since the composition of the fatty acid pool itself would be altered in response to temperature. This type of temperature-control mechanism has been shown to operate in a number of Bacilli (10-12). These bacteria, unlike E. coli but similar to higher organisms, produce unsaturated fatty acids directly from saturated analogs by the oxygen-dependent removal of two hydrogens from the hydrocarbon chain. The experiments reported in this paper show how temperature affects the relative rate of this transformation by controlling, in several interacting ways, the level of the desaturating enzyme in Bacillus megaterium.

EXPERIMENTAL PROCEDURE

The procedures used in these experiments are described in detail in the preceding paper (13). The results, however, are expressed in terms of desaturating enzyme units rather than in percent desaturation since it has been shown that, under standard assay conditions, percent desaturation is a linear function of enzyme activity (13). A desaturating enzyme unit is defined as that amount of enzyme which will convert 1% of the palmitic-1-¹⁴C acid incorporated by Bacilli cells into unsaturated fatty acid in 3 hours under standard assay conditions. For standard

conditions, all assays were carried out at a cell concentration of 5 g/liter in a buffer containing potassium phosphate, 0.05 M, pH 7.0; glucose, 10 g/liter and chloramphenicol, 100 mg/liter. The final temperature of the assay mixture was $20 \pm 1^{\circ}$ and the incubation was carried out at this temperature for 3 hours after the addition of palmitic-1- ^{14}C acid (0.25 μ moles/liter, 20 $\mu\text{c}/\mu$ mole). The workup of the assay mixtures was exactly as described in the previous paper (13).

RESULTS

The Induction of Desaturating Enzyme at 20° —The temperature-mediated induction of desaturating enzyme in B. megaterium was first demonstrated by experiments reported in the initial paper of this series (11) and examined in somewhat more detail in a recent preliminary note (14). Although the temperature-mediated induction of desaturating enzyme is a complex phenomenon and as such will be the sole topic of a future publication in this series, it would be useful to summarize certain characteristics of this process now. In previously published experiments (14) it was shown that in cultures of B. megaterium growing at 35° there is no desaturation, while in cultures growing continuously at 20° , the level of desaturating enzyme can be strictly correlated with growth rate of the cells. Even at maximal 20° growth rates however, the desaturating enzyme levels in cultures growing at 20° never approach those attainable by induction when cultures, growing at 35° , are transferred to 20° . The fact that chloramphenicol, added to a culture at the time of transfer from 35° to 20° completely blocks desaturation indicated that the appearance of

desaturating activity is directly related to protein synthesis and not to some activating effect of temperature on a pre-existing desaturase.

Typical induction curves for cultures transferred from 35° to 20° at various stages of growth are shown in Figure 1. As these curves suggest, the initial net rate of induction is relatively independent of growth phase although there is a short lag period and the rate declines somewhat in an early stationary-phase culture (Figure 1, Curve C). In contrast to initial net induction rates which are relatively constant, the maximal level of enzyme attained, as well as the time-point of this maximum, seems to be dependent upon the growth rate after transfer to 20° as well as the exact temperature of incubation. Since the assay system employed to measure levels of induced enzyme is no longer accurate when desaturating enzyme levels exceed 55 units (13), the maximum level reached after log-phase transfer (Curve A) was not determined. It must be emphasized that these curves represent net synthesis, not total synthesis. As will be shown later, the rate of enzyme inactivation is a function of temperature and proceeds according to first-order kinetics. Thus, the rate of inactivation of desaturating enzyme is proportional to the instantaneous concentration of the enzyme at any given temperature and if net synthesis is increasing at a linear rate with time, total synthesis must be increasing with an accelerating rate. This, of course, would be the case for the initial linear portions of the curves in Figure 1.

← FIGURE 1

Temperature-Mediated Irreversible Inactivation of Desaturating Enzyme

In the first paper of this series it was reported that when chloramphenicol was added to a culture of B. megaterium in which Δ^5 -desaturating enzyme

had been induced, desaturation levels rapidly decreased with time. It was concluded that the desaturase was subject to inactivation at 20° and that chloramphenicol, by blocking protein synthesis¹ and hence replenishment of inactivated enzyme, allowed the inactivation phenomenon to be reflected as a steadily declining level of desaturating activity. A large number of experiments have now verified this interpretation and, in addition have elucidated the kinetics of the inactivation reaction and the relationship between rate of inactivation and temperature. Figure 2 shows the in vivo inactivation rates of desaturating enzyme at 3 temperatures with the data plotted on a semi-logarithmic scale against time. Two characteristics of the inactivation process are immediately apparent from this data. First of all, the rates of inactivation are first-order; that is, the rate of inactivation of desaturating enzyme is proportional to the instantaneous concentration of the enzyme. Thus, the enzyme activity remaining (E) at any given time (t) is given by the equation $E = E_0 e^{-kt}$ where E_0 is the enzyme activity at $t = 0$ and k is a constant dependent on the half-life of the enzyme. The enzyme half-life ($t_{1/2}$) is independent of the initial enzyme concentration and can be readily determined graphically from curves such as those in Figure 2. Secondly, the temperature at which the cells are incubated with chloramphenicol is seen to profoundly affect the stability of the desaturating enzyme. At 12.5°, the desaturase from B. megaterium 14581-PC is stable. At 20.6 - 20.1°, it has a half-life of about 24 min while at 33.6 - 34.0°, the half-life has been reduced to 10 min. For these determinations, the standard desaturation assay procedure was used, employing 180 min incubations with palmitate-1-¹⁴C. However,

← FIGURE 2

when the 20° half-life was determined in triplicate, using assay times of 30, 60 and 180 min the $t_{1/2}$ values determined at 30 and 60 min were within 10% of the 180 min value. This result not only supports the validity of the assay procedure but confirms the conclusion that enzyme inactivation follows first-order kinetics.

Variations in Desaturase Half-Life at Temperatures near 20°—After repeating half-life determinations a number of times with B. megaterium 14581, it became obvious that at temperatures near 20° the in vivo stability of the desaturase varied widely with slight changes in temperature. This extreme sensitivity to temperature changes near 20° is illustrated by the data in Figure 3. The 2 curves represent half-life determinations of the desaturase from B. megaterium 14581-PC when the temperature was either kept absolutely constant at 20.0° during the course of the experiment (Curve A) or was allowed to drift downward from 21.5 to 19.6° (Curve B). These experiments demonstrate that perfectly linear curves describing first-order kinetics can be achieved for inactivation of the desaturase if the temperature is kept absolutely constant (Curve A, 20.0°) $t_{1/2}$ = 29 min) but that a temperature drift of less than 2° can cause a greater than 2-fold change in desaturase half-life (16 min at 21.5°; 37 min at 19.6°). Indeed, as Figure 2 clearly illustrates, a temperature drop of about 8° (from 20.6° to 12.5°) results in approximately a 60-fold increase in enzyme stability. On the other hand, slight temperature changes in the region between 30-35° have very little effect on desaturase half-life which, in any case is quite short ($t_{1/2}$ = 10-12 min) in this temperature range.

← FIGURE 3

Temperature-Mediated Inactivation of Desaturase in a Mutant Strain of *B. Megaterium*—When a culture of *B. megaterium* 14581 which had been maintained for more than one year by twice-monthly transfers on nutrient agar at room temperature (21-24°) was examined, it was found to possess a Δ^5 -desaturase with different half-life-temperature relationships than those typically observed with *B. megaterium* 14581. In addition, this strain, referred to henceforth as *B. megaterium*-old, showed different growth characteristics at 20° and 35°. The growth curves at these 2 temperatures for both *B. megaterium*-old and *B. megaterium* 14581 are shown in Figure 4, while desaturase inactivation curves at 3 temperatures for *B. megaterium*-old are shown in Figure 5. The growth curves show that *B. megaterium*-old grows more rapidly and to a higher cell density than does *B. megaterium* 14581 at 20°, while at 35° the situation is reversed. Although the relative differences in growth at the two temperatures are not great, they are probably sufficient to account for the emergence of a population consisting of *B. megaterium*-old if this strain was present either as a contaminant in the original culture or arose as a mutant in one of the early cultures. The bimonthly transfer process to fresh nutrient agar slants at room temperature would tend, of course, to select for the organism that grew most readily at this temperature. However, attempts to obtain *B. megaterium*-old or similar strains from other cultures of *B. megaterium* 14581 by repeated cycles of low-temperature growth and transfer have been unsuccessful.

The desaturase inactivation curves for *B. megaterium*-old (Figure 5) illustrate one interesting point. At temperatures between 19-35°, the

← FIGURE 4

← FIGURE 5

desaturase from B. megaterium-old is more stable to temperature-mediated inactivation than that from B. megaterium 14581 (compare with Figure 2) but at some lower temperature the situation is reversed. Thus, at 12.5°, the desaturase from B. megaterium 14581 is essentially stable ($t_{1/2} > 24$ hours) while at 8°, the desaturase from B. megaterium-old has a half-life of about 3 hours.

Desaturating Enzyme Synthesis at 33.8°—The experiments described thus far establish that the irreversible inactivation of desaturating enzyme proceeds according to first-order kinetics and that the rate of inactivation increases with increasing temperature. However, these experiments, carried out in the presence of chloramphenicol, give no information concerning the possible effects of temperature on the system that synthesizes the desaturating enzyme. Figure 6 shows the results of an experiment designed to determine whether desaturating enzyme is synthesized at 33.8°. At this temperature, of course, growing cultures of B. megaterium produce no unsaturated fatty acids and there is no net production of desaturating enzyme (a fact that can be inferred from the experiments already described). However, in the experiment described in Figure 6, desaturating enzyme synthesis was proceeding at an accelerating rate at 20° at the time the cells were transferred suddenly to 33.8°. The question to be answered then, concerns the fate, after this transfer, of the system which produces the desaturating enzyme. As curve B, Figure 6 shows, in the presence of chloramphenicol (i.e., in the absence of protein synthesis), desaturating enzyme, induced at 20°, is inactivated according to strict first-order kinetics (as expected) with a half-life of 17 min. In the absence of

← FIGURE 6

chloramphenicol however, (Curve A), the synthesis of desaturating enzyme proceeds at 33.8° for about an hour but at a steadily diminishing rate². After about 60 min, Curve A has attained the same slope as Curve B, indicating that synthesis of desaturating enzyme has ceased at 33.8°, even in the absence of chloramphenicol.

Net Synthesis of Desaturating Enzyme at 33.8° as a Function of Time—

From the data available in Figure 6 we can determine the net amount of desaturating enzyme synthesized during each 10 min interval along Curve A since experimental values are available for these time points. Net synthesis for any 10 min interval, beginning at time t is simply the difference between the observed value in enzyme units at time $t + 10$ and the value one would obtain if there were no enzyme synthesized during that interval³. This latter figure can be obtained graphically by extrapolating forward from the observed value at time t on Curve A to $t + 10$ along a line, the slope of which is given by the half-life curve (Curve B of Figure 6). By repeating this procedure for each 10 min interval, the net amount of enzyme synthesized during each interval can be determined. A more precise method, however, to determine this value makes use of the integrated form of the first-order rate equation for desaturase inactivation:

$$E/E_0 = e^{-kt} \quad (1)$$

where E_0 is the initial desaturating enzyme activity and E is the activity remaining at time t , assuming, of course, that there is no enzyme synthesis during this period. Since, from Curve B, Figure 6, $t_{1/2}$

for inactivation at 33.8° is 17 min, then at $t = 17$, $E/E_0 = 1/2$ and we are now able to solve for k in equation (1). The value of k is found to be 0.0407. We can now rearrange the equation to the form

$$E = E_0 e^{-0.0407t} \quad (2)$$

to solve for E at any time t . When $t = 10$, $E = 0.67E_0$. Thus, in the absence of synthesis, the amount of enzyme activity present at the end of any 10 min interval will be 0.67 multiplied by the activity found at the beginning of that interval. If we designate the activities on Curve A, Figure 6 at each 10 min interval as E_0 , E_{10} , E_{20} , etc. where the subscript denotes the time of measurement then the net synthesis, expressed in enzyme units, for the first 10 min interval is $E_{10} - 0.67E_0$, for the second 10 min interval, $E_{20} - 0.67E_{10}$, etc. These values for net synthesis are shown in Table I.

← TABLE I

Total Synthesis of Desaturating Enzyme at 33.8° as a Function of Time—To obtain the total enzyme synthesized during a 10 min interval (E_{tot}) rather than net enzyme synthesized (E_{net}) we must correct for that portion of newly-synthesized enzyme that is inactivated during the interval. For a first estimate, let us assume that, during any given 10 min interval, the rate of enzyme synthesis is constant. Thus, the amount of enzyme synthesized each min in a 10 min interval would be $1/10$ of the total synthesized in a 10 min interval, or $(0.1) E_{\text{tot}}$. However, enzyme synthesized during the first min undergoes inactivation for 10 min before it is measured while enzyme synthesized during the second min undergoes inactivation for only 9 min and so on. Thus, the contribution to E_{net} of enzyme

synthesized during any 1 min segment of the interval is dependent upon its time position within the interval. Since inactivation is described by a first-order rate equation whose terms are known, the contribution of the enzyme synthesized during each 1 min segment towards E_{net} can be calculated and is expressed by the term $(0.1) E_{tot} e^{-kt}$ where t is the time remaining in the 10 min interval after synthesis of $(0.1) E_{tot}$. The sum of the $(0.1) E_{tot} e^{-kt}$ terms for any 10 min interval is seen to equal E_{net} for that interval and we can write

$$E_{net} = (0.1) E_{tot} (e^{-10k} + e^{-9k} + \dots + e^{-k}) \quad (3)$$

Since k is known ($k = 0.0407$) we can solve for the e^{-kt} terms and rearrange to obtain

$$E_{tot} = 1.24 E_{net} \quad (4)$$

Since the values for E_{net} are known for each time interval (see Table I), the values for E_{tot} are easily calculated.

The Absolute Rate of Enzyme Synthesis at 33.8° as a Function of Time

Although the values for E_{net} at each time interval are directly derived from experimental values, E_{tot} is an estimate based on the assumption that the rate of enzyme synthesis is a constant during each 10 min time interval. This assumption is obviously not correct. However, because of the relatively large number of data points, a good estimate of the instantaneous rate of enzyme synthesis at any time can be obtained by taking the average rate for any 10 min interval to be simply $E_{tot}/10$ and then plotting the $E_{tot}/10$ values against time by locating them at the

midpoint of each interval. These points will then define a curve which gives the instantaneous rate of desaturase synthesis at any time. A more precise method, however, makes use of the fact that enzyme synthesis terminates at about 60 min (i.e., when the slope of Curve A, Figure 6 becomes the same as that of Curve B). We also know, from either the data for E_{net} (Table I) or from plotting the $E_{\text{tot}}/10$ values as described above, that enzyme synthesis is actually decreasing in a linear fashion during the 60 min period of synthesis. Thus, the amount of enzyme synthesized each min (E') is accurately expressed by the equation:

$$E' = E'_0 \left(\frac{60 - t_s}{60} \right) \quad (5)$$

Where E'_0 is the amount of enzyme synthesized in the first min and t_s is the time at which synthesis of E' began. To fit the data, this equation can be changed slightly and used to determine the amount of desaturating enzyme synthesized in the first min of each 10 min interval and thus the rate of synthesis (in enzyme units per min) at the beginning of each 10 min interval. Thus, the amount of enzyme (E) synthesized during any 1 min of a 10 min interval is given by

$$E = E_0 \left(\frac{t_0 - t_s}{t_s} \right) \quad (6)$$

Where E_0 is the amount of enzyme synthesized during the first min of the interval, t_0 is the time remaining from the start of the interval to the time (60 min) when all synthesis has ceased (i.e., for the first 10 min

interval, $t_0 = 60$, for the second, $t_0 = 50$, etc.) and t_s is the elapsed time from the start of the synthesis of E within the interval to the end of the interval (i.e., t_s goes from 0 to 9). Now, by the same reasoning used to derive equation (3) we see that the contribution of E to E_{net} is given by the term

$$E_0 \left(\frac{t_0 - t_s}{t_0} \right) e^{-kt} \quad (7)$$

where t has the same meaning as in equation (3), that is, the time remaining in the 10 min interval after the synthesis of E begins. Thus, while t_s goes from 0 to 9, t goes from 10 to 1. We can now write:

$$E_{net} = E_0 \left[\left(\frac{t_0 - 0}{t_0} \right) e^{-10k} + \left(\frac{t_0 - 1}{t_0} \right) e^{-9k} + \dots + \left(\frac{t_0 - 9}{t_0} \right) e^{-k} \right] \quad (8)$$

Since all the terms but E_0 are known, we can rearrange and solve for E_0 , the amount of desaturating enzyme synthesized during the first min of each 10 min interval. Since E_0 is actually the rate of synthesis of desaturating enzyme (enzyme units per min) we have thus measured the absolute rate of desaturating enzyme synthesis as a function of time and these results are shown in Figure 7. The maximal rate of synthesis (at 0-time) is about 1.8 desaturating enzyme units per min. This rate of synthesis is comparable to the rate (estimated in the same manner) at 20° at the time of transfer⁴. More interesting however, is the fact that enzyme synthesis at 33.8° begins to decrease at a constant rate immediately after transfer to 33.8°. In other words, although desaturating enzyme itself undergoes inactivation according to first-order kinetics, the system which

← FIGURE 7

synthesizes the enzyme decays with zero-order kinetics.

Desaturation at 33.8°—Table II compares the progress of palmitate desaturation at 33.8° with desaturase inactivation at the same temperature. Two points should be noted: First of all, palmitate can be desaturated at 33.8°. Indeed, the initial rate of desaturation appears to be as rapid or more so than the comparable rate at 20° with the same amount of enzyme. Secondly, the rate of desaturation decreases rapidly with time, much more so than can be accounted for by the rate of irreversible inactivation of enzyme. These data suggest but do not prove that there may be a reversible inactivation of desaturase at 33.8° (that is, reversible by changing the temperature to 20°).

← TABLE II

DISCUSSION

The results of the experiments reported in this paper demonstrate that unsaturated fatty acid biosynthesis in Bacillus megaterium is strongly influenced by relatively small changes in temperature. Indeed, it seems likely from the results reported here that temperature is the dominant controlling factor in the regulation of the ratio of unsaturated to saturated fatty acids in this organism⁵. Temperature changes do not seem to have a significant effect on the rate of the desaturation reaction itself; that is to say, at a given concentration of desaturating enzyme, the initial rate of palmitate desaturation is relatively temperature-independent. Rather, temperature acts in several distinct ways to regulate the level of the desaturating enzyme and thus to indirectly control the level of unsaturated fatty acid produced. At least 3 temperature-mediated

mechanisms which affect the level of desaturating enzyme have been elucidated. One, enzyme induction or hyperinduction, is observed when a culture, growing at a relatively high temperature (32-35°) is transferred to a low temperature (20°). A second mechanism, zero-order decay of the desaturase synthesizing system, is observed when a culture that has been incubated at 20° long enough to induce a high rate of desaturase synthesis, is transferred back to 34°. A third temperature-mediated mechanism, irreversible first-order inactivation of the desaturating enzyme, can be observed to operate at all temperatures but it becomes increasingly more important as a regulatory factor as temperature increases. The following discussion of each of these 3 mechanisms will show how they interact to regulate both the instantaneous and steady state levels of Δ^5 -desaturase in B. megaterium.

1. Irreversible inactivation of desaturating enzyme - as Figures 2, 3, 5 and 6 clearly show, the desaturase of B. megaterium undergoes an irreversible inactivation at a rate which is determined solely by temperature. In addition, at all temperatures the kinetics of inactivation are first-order (i.e., the rate of inactivation of desaturating enzyme is described by the exponential decay law and is proportional to the instantaneous concentration of enzyme). In this respect, desaturase inactivation is strictly analogous to radioactive decay or, more relevantly, to a typical thermal decomposition such as that of nitrogen peroxide (16). One can rule out inactivation of the desaturase by a proteolytic enzyme synthesized in response to temperature since the kinetic data does not accommodate such an interpretation. Furthermore, the fact that both the kinetics and the

rate constants for inactivation are the same in either the presence or absence of chloramphenicol (Figure 6) support the conclusion that inactivation of desaturase is brought about by a direct temperature-activated, irreversible denaturation of the desaturase protein.

The remarkable effect that a 2° change in temperature can have on the rate of desaturase inactivation is clearly illustrated in Figure 3. Here, a change from 21.5° to 19.6° results in a more than 2-fold increase in desaturase half-life. Obviously, the level of desaturating enzyme at a given temperature is a function both of enzyme synthesis and enzyme inactivation and, at temperatures near 20° the rate of inactivation seems to be much more responsive to slight temperature changes than is the rate of synthesis. Since the absolute rate of inactivation at moderate desaturase levels⁶ can equal or exceed the absolute rate of synthesis at temperatures of 20° or above, it is apparent that desaturase inactivation is an extremely sensitive and important factor in determining the overall rate of unsaturated fatty acid biosynthesis.

2. Enzyme induction - When a culture of *B. megaterium*, growing at 35° , is transferred to 20° , desaturating enzyme activity is rapidly induced after a short lag period and appears to increase in a relatively linear fashion for at least 1 hour (Figure 1). The fact that chloramphenicol, added at any given time after transfer, completely blocks the further increase of desaturase activity shows that protein synthesis is necessary for the induction of the desaturating enzyme and that no active desaturase is present at the time of transfer from 35° . The relatively linear increase in desaturase activity during the initial phase of induction can

be somewhat misleading, in the absence of other data, in assessing the kinetics of induction. However, once it is recognized that desaturase inactivation is proceeding simultaneously but at an absolute rate that increases with increasing enzyme concentration (see footnote 6) it is obvious that, although net enzyme synthesis during induction may proceed at a relatively constant rate, the absolute rate of desaturase synthesis during the first hour of induction must be proceeding at an accelerating rate to maintain the observed linearity of net increase in the face of an accelerating rate of inactivation. With this in mind, the induction data (Figure 1) are seen to be consistent with the view that the desaturase synthesizing system is not present in active form at the time of transfer from 35° to 20° but must itself be induced. This interpretation is supported by the observation that, when a fully induced culture is transferred back to 34°, not only does the desaturase undergo more rapid first-order decay as expected but the system which produces the desaturase also decays (but with zero-order kinetics - see below).

3. Decay of the desaturase synthesizing system - As Figure 7 shows, the system which produces the desaturating enzyme at 20° is not stable at 34° but rather the rate of production of desaturase decreases to zero over the period of 1 hour. This decrease is linear with time (i.e., the desaturase synthesizing system decays with zero-order kinetics). Such kinetics would appear to rule out the simple thermal inactivation of some component of the synthesizing system and would also be inconsistent with a temperature-mediated change in the binding constant between components in the system (between messenger RNA and ribosomes, for example).

One could speculate that a repressor is synthesized at 34° which binds to the DNA site responsible for the synthesis of the desaturase messenger RNA. If this were the case, then the induction of desaturase at 20° may represent a de-repression. However, there are certainly a number of other possible explanations consistent with the limited data available at present and further studies of both the decay and the induction of the desaturase synthetase system are necessary before one can distinguish among the various alternatives.

4. Other possible temperature effects on desaturation - The results shown in Table II suggest that a temperature-reversible inactivation of desaturase may occur at 34° . As the data show, the initial rate of palmitate desaturation at this temperature is comparable with the observed rate at 20° but desaturation falls off more rapidly at 34° than can be accounted for by irreversible inactivation of the desaturating enzyme.

This is evidence for a rapid but temperature-reversible (by transfer back to 20°) inactivation of desaturase which is superimposed on the slower but irreversible first-order inactivation. At present, however, this conclusion is tentative since alternate explanations are possible that are consistent with the data.

Another phenomenon which may or may not be temperature-mediated is the eventual decline in net desaturating enzyme synthesis in hyperinduced cultures (see Figure 1 and also Reference 14). The mechanism by which this is accomplished is not yet clear nor is it yet possible to accurately predict at what point the decline in net synthesis will begin. The process appears to be quite complex and the induction curves shown in Figure 1

are typical but certainly not inclusive. At times we have noticed a decline in net synthesis followed by a second maximum but again the mechanism remains obscure. Nevertheless, at least 3 important temperature-mediated controls on unsaturated fatty acid biosynthesis in B. megaterium have been elucidated. Acting together, they represent an extremely sensitive and versatile system for regulating the level of unsaturated fatty acids in response to long or short-duration changes in environmental temperature.

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FOOTNOTES

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1. The concentration of chloramphenicol (100 mg/liter) routinely used in these experiments is about 5 times the concentration needed to completely block the incorporation of tryptophan-U-¹⁴C into B. megaterium protein as measured by trichloroacetic acid precipitable radioactivity (A. Fulco, unpublished experiment).
 2. It is only the comparison of Curve A with Curve B (the inactivation curve for desaturase in the total absence of protein synthesis) that allows us to conclude that there is synthesis of desaturase at 33.8° when chloramphenicol is not present. As long as the slope of curve A is greater than that of Curve B, there must be some synthesis of enzyme to partially compensate for the loss of enzyme caused by irreversible inactivation. When desaturase synthesis totally ceases, the slope is then determined only by the rate of inactivation as given by Curve B and the slopes of Curves A and B must now be the same.
 3. Note that the expression "net synthesis" as defined here has a slightly different meaning than that often associated with the term.

4. An exact determination of the absolute rate of enzyme synthesis at 20° at the time of transfer to 33.8° could not be made because an induction curve at 20° was not obtained for this experiment. However, based on induction curves from other experiments, the rate of synthesis after 60 min at 20° is between 1.4 and 1.8 desaturating enzyme units per min.
5. At any given temperature the rate of unsaturated fatty acid biosynthesis seems to be proportional to growth rate (i.e., rate of membrane formation) but the proportion of unsaturated to saturated fatty acids throughout the growth cycle remains relatively constant. It thus seems apparent that growth controls the rate of total fatty acid biosynthesis but does not affect the relative rates of unsaturated and saturated fatty acid production.
6. It must be remembered that the absolute rate of irreversible inactivation at any given temperature is a function of the instantaneous enzyme concentration (see equations (1) and (2) in the text). Thus, as the enzyme concentration increases, the inactivation reaction becomes progressively more important in determining the net rate of desaturase production.

LEGENDS FOR FIGURES

Fig. 1. Temperature-mediated induction of Δ^5 -desaturating enzyme in B. megaterium 14581. In 3 separate experiments, cultures of B. megaterium were grown at 35° in the usual way to various cell densities and then cooled rapidly in ice to 20° and transferred to a 20° shaker. At appropriate time intervals after transfer, 5 ml volumes of culture were withdrawn and assayed by the usual procedure to determine the levels of induced Δ^5 -desaturating enzyme. In the first experiment (curve A) transfer from 35° took place at a cell concentration of 6.7 g/liter. After 120 min at 20°, the cell density was 10.5 g/liter. In the second experiment (curve B), transfer took place at 11.2 g/liter and reached 13.8 g/liter after 180 min at 20°. In the last experiment (curve C) the cells were transferred at 13.0 g/liter and there was no further growth after 120 min at 20°.

Fig. 2. Temperature-mediated inactivation of desaturating enzyme in a pure culture of B. megaterium 14581. A pure culture of B. megaterium 14581 was prepared by the streak-plate method (15) from a late-log phase shake culture growing at 35°. The nutrient agar plate was incubated overnight at 35° and a single colony was removed with a wire loop, streaked on a nutrient agar slant and incubated for several days at 35°. Cells from this slant are referred to as B. megaterium 14581-PC and were used as an inoculum for the shake culture used in this experiment.

B. megaterium 14581-PC was grown at 35° to a cell density of 6.8 g/liter, cooled rapidly to 20° by swirling in an ice bath, and then incubated with shaking at 20° for 68 min to induce desaturating enzyme. The cells (now 8.7 g/liter) were harvested by centrifugation and divided into 3 portions. Each portion was resuspended in phosphate-glucose-chloramphenicol assay buffer of the appropriate temperature to a concentration of 10 g/liter. Waring blenders, equilibrated to buffer temperatures, were used to carry out the resuspensions. Portion A was resuspended at 33.6°, portion B at 20.6° and portion C at 12.5°. Immediately after resuspension, each portion was transferred to a shaker (held at the temperature of the resuspension buffer) and 5 ml samples were withdrawn at intervals and pipetted into 5 ml of assay buffer. Palmitic-1-¹⁴C acid (2.5 μ moles) was added immediately and the assay procedure for desaturating enzyme was carried out in the usual manner. Since the assay incubations were all done under standard conditions (20°) it was necessary to preheat or precool the assay buffer solutions so that, when mixed with the samples containing the cells from the 12.5° or 33.6° incubations, a temperature of $20 \pm 1^\circ$ was obtained in the assay vessel after mixing. After the last sample was withdrawn from each flask, the incubation temperature was again measured. Portion A was 34.0° (an increase of 0.4° over the starting temperature), portion B was 20.1° (a drop of 0.5°) and portion C was 12.5° (no change). The values obtained for desaturating enzyme levels are plotted on a semi-log scale against time.

Fig. 3. Variations in the desaturase half-life at temperatures near 20°.

In experiment A, B. megaterium 14581-PC was grown at 34-35° to a density of 8.4 g/liter, cooled rapidly in ice to 20° and then incubated with shaking at 20° for 60 min to induce the desaturating enzyme. At the end of this period the cells (10.1 g/liter) were harvested by centrifugation, resuspended (10.0 g/liter) at 20.0° in assay buffer and the rate of inactivation of the desaturating enzyme determined as described under Fig. 2. Both the starting temperature and the final temperature (after 1 hour) measured exactly 20.0°. In experiment B, B. megaterium 14581-PC was grown at 34-35° to 9.2 g/liter, cooled to 20° in ice and incubated for 70 min at 20° to induce desaturase. The cells (10.3 g/liter) were harvested by centrifugation, resuspended (10g/liter) in assay buffer at exactly 21.5° and then transferred to the shaker which was maintained at about 19°. During the course of incubation (90 min) the temperature of the buffer containing the resuspended cells gradually drifted downward so that the final temperature was 19.6° (a drop of 1.9°). The rate of inactivation of desaturating enzyme was determined in the usual way and the values obtained for desaturating enzyme levels are plotted on a semi-log scale against time. Curve A was obtained for the inactivation study carried out at exactly 20.0°. Curve B was obtained for the temperature range 21.5° - 19.6°.

Fig. 4. Relative rates of growth of 2 strains of B. megaterium at 20° and 35°. B. megaterium-old (curves A and A') and B. megaterium 14581 (curves B and B') were grown in shake culture at 20° and 35°.

For 20° growth, each of 2 flasks containing 1 liter of medium was inoculated with 50 ml of log-phase starter culture of the appropriate strain. The flasks were then incubated at 20° for 24 hours and growth was followed by withdrawing 10 ml samples at intervals for reading in a Klett colorimeter. The relatively large inoculum was required to eliminate the long and somewhat variable lag period observed at 20° when small inocula are used. The 35° growth curves were obtained in the same way except that 5 ml of starter culture were used as inocula for each flask.

Fig. 5. Temperature-mediated inactivation of desaturating enzyme in a mutant culture (B. megaterium-old) - A culture of B. megaterium-old was grown at 33° to late log phase (9.0 g/liter) and then cooled rapidly in ice to 20° and incubated at this temperature for 70 min to induce desaturating enzyme. At the end of this period the cells (now 11.0 g/liter) were harvested by centrifugation, divided into 3 portions and resuspended at a concentration of 10 g/liter in assay buffer as described under Fig. 2. The inactivation rates were then determined as described under Fig. 2 at temperatures of 32.5° (curve A), 19.5° (curve B), and 8.0° (curve C). Final temperatures were within 0.5° of the starting temperatures. The values obtained for desaturating enzyme levels are plotted on a semi-log scale against time.

Fig. 6. The effect of temperature on the desaturase synthesizing system.

B. megaterium-old was grown at 34° to a density of 9.2 g/liter, cooled rapidly in ice to 20° and then incubated at 20° for 60 min to induce desaturating enzyme. At the end of this period the cells (11.2 g/liter) were harvested by centrifugation and suspended in 0.05 M potassium phosphate buffer, pH 7.0 containing 10 g/liter of glucose. The buffer temperature, after mixing with cells at a concentration of 10 g/liter, was 33.8° . After mixing, the cell suspension was divided into 2 equal portions (A and B). Both portions were placed in the incubator-shaker at 33.8° and chloramphenicol (100 mg/liter) was added to portion B to stop protein synthesis. Samples were withdrawn from both portions at intervals and assayed for desaturating enzyme as described under Fig. 2. The temperature of both portions was maintained at 33.8° throughout the course of the experiment. Curve A shows the levels of the desaturating enzyme at 33.8° in the absence of chloramphenicol while curve B shows the typical first-order irreversible inactivation of enzyme in the presence of chloramphenicol. The data are plotted on a semi-log scale against time.

Fig. 7. The rate of desaturating enzyme synthesis at 33.8° as a function of time. The curve above was obtained by plotting E_0 values calculated by the use of equation (8) as described in the text.

TABLE I

The net synthesis of desaturating enzyme at 33.8°

The net synthesis of desaturating enzyme for each 10 min time interval was calculated from the equation $E_{net} = E(t + 10) - 0.67 E_t$ where $E(t + 10)$ is the measured desaturating enzyme activity at the end of a 10 min interval and E_t is the enzyme activity at the beginning of the interval. The derivation of this equation is explained in the text.

Time Interval	$E(t + 10)$	$0.67 E_t$	E_{net}
<u>min</u>	<u>desaturating enzyme units</u>		
0-10	46.6	33.6	13.0
10-20	42.0	31.2	10.8
20-30	36.2	28.1	8.1
30-40	29.5	24.3	5.2
40-50	23.0	19.8	3.2
50-60	17.0	15.4	1.6
60-70	11.3	11.4	-0.1

TABLE II

Desaturation of Palmitate-1-¹⁴C at 33.8°

Two portions of B. megaterium-old from the experiment described under Fig. 6 were resuspended in the phosphate-glucose-chloramphenicol assay buffer at 33.8° after induction of desaturase at 20°. One portion was resuspended at a concentration of 10 g/liter and was used for the determination of desaturase half-life at 33.8° by the usual assay method. The second portion of cells was resuspended to give 5 g/liter. Palmitic-1-¹⁴C acid (67 μ moles) was added to 250 ml of the cell suspension which was then incubated at 33.8°. Samples were withdrawn at intervals, the cells were harvested by millipore filtration and total desaturation of palmitate-1-¹⁴C was determined in the usual way.

Incubation time at 33.8°	Desaturating enzyme level	Total desaturation (cumulative)
<u>Min</u>	<u>desaturating enzyme units</u>	<u>%</u>
0	51.0	0
10	33.5	11.0
20	21.5	13.5
40	9.5	14.7
60	4.0	14.7
90	2.5	14.7

Fig 2

Fig 3

Fig 7