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Biosynthesis of Unsaturated Fatty Acids by Bacilli

HYPERINDUCTION AND MODULATION OF DESATURASE SYNTHESIS*

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Significant relationships have been established between the initiation and rate of fatty acid Δ^5-desaturase synthesis in *Bacillus megaterium* ATCC 14581 and controlled perturbations in culture temperature, cell growth, protein synthesis, and RNA synthesis. *B. megaterium* growing from inoculum at 35° contained neither unsaturated fatty acids nor the Δ^5-desaturase responsible for their production. When the culture temperature was lowered rapidly to 20°, synthesis of desaturase began within 5 min, attained a maximum rate at about 15 min, and continued at this high rate for up to 90 min after the shift to 20°. This "hyperinduction" process, so-called because the rate of desaturase synthesis after culture transfer from 35° to 20° far exceeded the rate found in comparable cultures growing from inoculum at 20°, was dependent on protein synthesis and RNA synthesis initiated after transfer. Rifampicin, added at the time of culture transfer, completely blocked the appearance of desaturase activity. The maximum rate of desaturase synthesis achieved during hyperinduction, when normalized for the concurrent rate of protein synthesis, was always a constant, regardless of experimental conditions. The hyperinduction phase was followed by a period of rapid attenuation of desaturase synthesis until the rate was at or below that in comparable cultures grown at 20° from inoculum. Experimental evidence suggests that the turn-off of hyperinduction at 20° in transfer cultures as well as the relatively low rate of desaturase synthesis in cultures growing from inoculum at 20° resulted from the action of a temperature-sensitive modulator protein which was absent in 35° cultures but was produced at 20°. Modulation of desaturase synthesis at 20° could temporarily be eliminated by pulsing a culture at 35° for 30 min and could be permanently abolished by a level of chloramphenicol sufficient to cause an 80% inhibition of overall protein synthesis. It was concluded that the rate of desaturase synthesis during hyperinduction (*i.e.* in the presumed absence of modulator) was strictly proportional to the rate of overall protein synthesis, while modulation of desaturase synthesis appeared to be an exponential function of protein synthesis. The data were consistent with the hypothesis that the active modulator was an oligomeric protein in equilibrium with an inactive monomeric precursor and that the modulator may act at the level of transcription by selectively inhibiting the synthesis of the messenger RNA coding for the desaturase.

Almost all poikilothermic organisms show an increasing proportion of unsaturated to saturated fatty acids in their membrane lipids as environmental temperature decreases. There seems little doubt that this inverse relationship represents an adaption on the part of these organisms to control membrane fluidity and hence to maintain membrane integrity and function in the face of temperature fluctuations (1-3). There are various ways by which the relative level of unsaturated fatty acids in biological membranes can be regulated in response to temperature changes. These include temperature-induced changes in the relative rates of incorporation of saturated and unsaturated fatty acids into membrane lipids (4), temperature-mediated changes in the concentration of desaturation cofactors (such as O_2 in the oxygen-dependent pathway) which in turn could affect the rate of unsaturated fatty acid biosynthesis (5-8), or the effects of temperature on the stability or rate of synthesis of the desaturase itself. The last mentioned type of regulation has been shown to operate in a number of bacilli including *Bacillus megaterium* which desaturates palmitate *in vivo* to *cis*-5-hexadecenoate (9-18). In *B. megaterium* ATCC 14581, at least three control mechanisms were demonstrated which regulated 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 directly responsive to temperature changes was the irreversible inactivation of desaturating enzyme. This inactivation, *in vivo*, followed first order kinetics at all temperatures, and the enzyme half-life was determined solely by the incubation temperature (15, 17). A second control process was that of desaturase induction (9, 10). *B. megaterium* cultures growing at 35° did not contain unsaturated fatty acids nor did they show desaturase activity. When these cultures were transferred to 20°, however, synthesis of desaturase began within 5 min and continued at a high rate for about 1 h (13). This "hyperinduction" process, so-called

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because the levels of desaturating enzyme formed in cultures after transfer from 35° to 20° far exceeded the levels found in cultures growing normally at 20°, was completely blocked by the addition of the protein synthesis inhibitor, chloramphenicol, added before or at the time of transfer. A third process, the zero order decay of the desaturase-synthesizing system, was observed when hyperinduced cultures were transferred from 20° back to 35°. After about 1 h at 35°, these cultures no longer produced detectable amounts of desaturating enzyme (15). A similar process seemed to occur in cultures transferred from 35° to 20°. There was an eventual decline in desaturase synthesis in hyperinduced cultures, but the mechanism by which this was accomplished was unknown, and it was not possible to predict at what point the decline would begin. On the basis of preliminary evidence, however (15, 18), it was suggested that the rapid decrease in desaturase synthesis following hyperinduction at 20° was caused by the synthesis of a repressor.

We now wish to report the results of experiments that characterize the details of the hyperinduction process and the subsequent decrease in desaturase synthesis and that lead to some understanding of the temperature-mediated mechanisms responsible for these phenomena.

EXPERIMENTAL PROCEDURE AND CALCULATIONS

Growth and Incubation of Bacteria—*Bacillus megaterium* ATCC 14581 was grown on a salts/glucose/casamino acids medium in liquid shake culture as previously described (10). Unless noted otherwise, each experiment was begun by pouring 1 liter of shake culture into a 2-liter conical flask equipped with an overhead stirrer and immersed in a water bath of the appropriate temperature. After transfer, stirring was begun and continued at a rapid, constant rate throughout the course of incubation. Under these conditions, approximately 6 min were required for a culture shifted from 35° to a water bath at 20.0° to reach a temperature of 20.1°. Unless noted otherwise, the initial ("zero time") samples were taken from cultures exactly 6 min after transfer. Cell concentrations were determined by optical density readings in a Klett-Summerson photoelectric colorimeter through a 54 (green) filter and are expressed in Klett units (KU). A reading of 100 KU corresponded to a cell concentration of about 2.5 g/liter on a wet weight basis.

Substrates and Inhibitors—[1-^{14}C]Palmitic acid, [2-^{14}C]uracil, [*methyl*-^{14}C]thymidine, and [3-^{14}C]tryptophan were purchased from New England Nuclear. The labeled palmitate had a radiopurity of 98% as determined by gas radiochromatography, while the other ^{14}C-substrates had radiopurities of 99% as assayed by radioscanning on paper chromatograms. Chloramphenicol and nalidixic acid were purchased from Sigma Chemical Co., while rifampicin, B grade, was obtained from Calbiochem. 6-(*p*-Hydroxyphenylazo)-uracil was the generous gift of Dr. Bernard Langley, Imperial Chemical Ind., Ltd., Macclesfield, England.

Assay for Desaturating Enzyme—The assay for desaturating enzyme has been described in detail previously (14, 17) but basically involves the incubation of 5 ml of *B. megaterium* culture with 5 ml of a solution containing phosphate buffer, glucose, chloramphenicol, and 2.5 nmol (2×10^5 cpm) of [1-^{14}C]palmitic acid for 3 h at 20° ± 0.2°. A desaturase unit (DU) is defined as that amount of desaturase that will convert 1% of the label recovered in fatty acids to *cis*-5-hexadecenoate under these standard conditions. At high levels of desaturation, a correction factor must be applied to the percentage of desaturation value to obtain actual desaturase units. This factor is determined by extrapolation back along the half-life curve as described previously (7).

Calculation of Total Desaturase Synthesis—As we showed previously (15), the rate of inactivation of desaturating enzyme, *in vivo*, is always strictly first order and can be expressed in terms of a half-life ($t_{1/2}$) which is a constant at a given incubation temperature. The amount of desaturase (E) remaining at the end of an interval of length T in the absence of desaturase synthesis during the interval is given by the integrated form of the first order rate equation

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

where E_0 is the amount of desaturase present at the beginning of the interval, and k is a constant derived from the desaturase half-life and is equal to $\ln 0.5/t_{1/2}$. When desaturase synthesis occurs during the interval, the amount of desaturase present at the end of the interval (E_T) will be greater than E. The net amount of desaturase (E_{net}) synthesized during the interval is simply equal to $E_T - E$ or, from Equation 1

$$E_{net} = E_T - (E_0)(e^{-kT}) \quad (2)$$

To determine the total amount of desaturase (E_{total}) synthesized during the interval, we must correct for that portion of the newly synthesized enzyme that was inactivated during the interval. If we make the assumption that enzyme synthesis proceeded at a constant rate during the interval, then total desaturase synthesis is given by Equation 3.

$$E_{total} = E_{net}/e^{-kT/2} \quad (3)$$

Since, from Equation 2, E_{net} can be expressed in terms of E_0 and E_T, Equation 3 can be rewritten as follows:

$$E_{total} = E_T/e^{-kT/2} - (E_0)(e^{-kT/2}) \quad (4)$$

Equation 4 can now be used to calculate E_{total}, in desaturase units, directly from the experimental data. The assumption that enzyme synthesis takes place at a constant rate during a given interval is not, of course, always true. To the extent that it is not, the real E_{total} may be more or less than the calculated value. However, for measurements over short intervals at 20° (where $t_{1/2}$ = 27 min) the calculated values are quite accurate. For example, if measurements were taken at 5-min intervals and all new desaturase synthesis actually occurred instantaneously at the beginning of each interval, the calculated values (from Equation 4) would be 6.2% too low. Conversely, if all synthesis of enzyme took place at the end of an interval, the values calculated would be 6.6% too high. The corresponding errors for these theoretical extremes would be 12% and 13.7%, respectively, for data points at 10-min intervals and 32% and 47% for sampling at 30-min intervals.

Protein Synthesis Assay—The rate of protein synthesis in bacilli culture was determined by measuring the incorporation of [3-^{14}C]tryptophan into trichloroacetic acid-precipitable material under standard conditions as described in detail previously (17). All protein synthesis rates are given in relative terms to facilitate comparison of one experiment with another and to simplify the calculation of the normalized rates of desaturase synthesis (see below). In practice, a standard rate value was obtained by measuring the rate of protein synthesis in a culture that had attained a cell density of 100 KU after growing at 20° in shake culture from inoculum. This standard value (15,000 cpm/min/100 KU) was then divided into all other experimental values (expressed in the same units) to obtain the relative rate values in terms of the dimensionless quantity, P.

Normalized Rate of Desaturase Synthesis—The average rate of desaturase synthesis during any interval is simply E_{total}/T where E_{total} is given in desaturase units (DU) and T, the span of the interval, is given in minutes. However, it was found that significant correlations could be established among various experiments if the rate of desaturase synthesis were expressed as a function of the relative rate of protein synthesis. This so-called "normalized" rate of desaturase synthesis is given by the term $E_{total}/T/P$ where P is obtained by averaging the relative rates of protein synthesis at the beginning and end of the measured interval.

RNA Synthesis Assay—The rate of RNA synthesis in bacilli cultures was estimated by measuring the incorporation of [2-^{14}C]uracil into trichloroacetic acid-precipitable material by means of a modification of the procedure described by Coote *et al.* (19). Incorporation was initiated by mixing 1 ml of culture with 1 ml of glucose/phosphate buffer (pH 7.0) containing 50 μM uracil (0.56 μCi/ml). The sample tube was then shaken in a water bath for 10 min at the same temperature as the bulk culture. Incorporation was terminated by the addition of cold 5% trichloroacetic acid solution containing 100 μg/ml of unlabeled uracil. The sample was then allowed to stand in ice for 30 min before collecting the precipitate on a Millipore depth filter (AP 25). The precipitate was washed on the filter with a total of 10 ml of cold trichloroacetic acid/uracil solution and finally with 10 ml of cold 1:1 (v/v) diethyl ether:ethanol. The filters were then dried at 70° and their radioactivity determined by liquid scintillation counting. The rate of RNA synthesis was expressed as counts per min per 100 KU and was corrected for zero time controls. Incorporation of [2-^{14}C]uracil was linear during the 10-min incubation period.

DNA Synthesis Assay—The assay for DNA synthesis was carried out as described above for measuring RNA synthesis except that

[*methyl*-^{14}C]thymidine was used as a substrate.

Counting Procedures—Radioactivity determinations were carried out by liquid scintillation counting as previously described (14).

RESULTS

Temperature-triggered Hyperinduction of Desaturase Activity—The results shown in Figs. 1 to 3 clearly illustrate the nature of the hyperinduction phenomenon. A culture growing from inoculum at 20° maintains a relatively constant level of desaturase activity throughout the major portion of its growth period (Fig. 1, *Curve A*). On the other hand, when a culture growing at 35° (no detectable desaturase activity) is transferred to 20°, desaturase activity is rapidly hyperinduced (Fig. 1, *Curve B*) and increases to a level 4 to 5 times that of the control culture growing at 20° from inoculum. The activity in the hyperinduced culture reaches a peak within 1 h after transfer and then drops rapidly to a level at or below that found in the control culture. The effect of transfer from 35° to 20° on culture growth and the rate of protein synthesis is illustrated in Fig. 2. The downward temperature shift typically results in an initial 40 to 60% inhibition of overall protein synthesis as measured by [3-^{14}C]tryptophan incorporation (Fig. 2, *Curve B*) when compared either to protein synthesis in the same culture just before transfer or to the control culture at the same stage of growth (Fig. 2, *Curve A*). In the experiment shown, this relative inhibition is gradually overcome until, at 4.5 h, the protein synthesis rates in the transfer and control cultures coalesce.

For more revealing comparisons between the two cultures, the actual rates of desaturase synthesis in the two cultures were determined, and these rates were then normalized for the rates of overall protein synthesis (Fig. 3). Under the same growth and transfer conditions the results shown in Fig. 3 were always quite reproducible. We consistently observed, during growth of a culture at 20° from inoculum, that the normalized rate of desaturase synthesis increased with decreasing protein synthesis, but in an undulating manner as shown in Fig. 3. When such a culture entered early stationary phase (usually near a cell density of 500 KU), the normalized rate of desaturase synthesis reached a maximum of 5 to 7 DU/min/*P* and then began to decline. Generally, determinations of

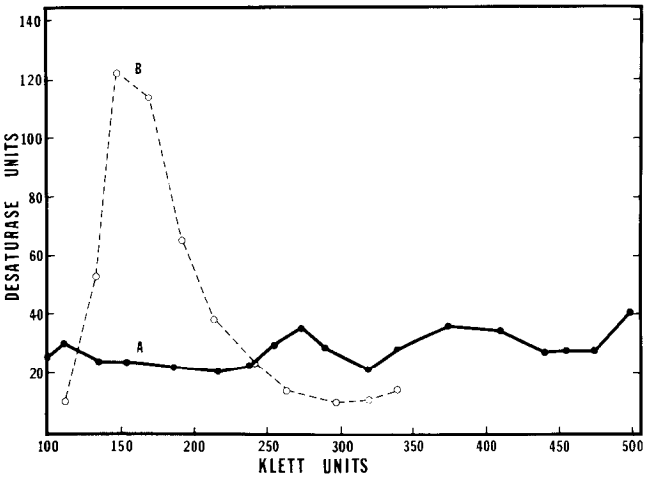

FIG. 1. Levels of desaturase activity in *Bacillus megaterium* cultures at 20°. In the first experiment, a 1-liter culture was grown from inoculum at 20° ± 1° in an incubator shaker. Desaturase activity was determined at approximately 30-min intervals (Curve *A*) during growth from 100 to 500 KU over a period of 8 h. In the second experiment, a 1-liter culture was grown from inoculum at 35° ± 2° in an incubator shaker until cell density reached 112 KU. The culture was then transferred to a 20.0° water bath and, with rapid stirring, was incubated for 5 h at this temperature. Desaturase activity (Curve *B*) was determined at 30-min intervals throughout this period. In a control experiment (not shown) no desaturase activity was detected at any time in a culture grown from inoculum to 450 KU at 35° ± 2°.

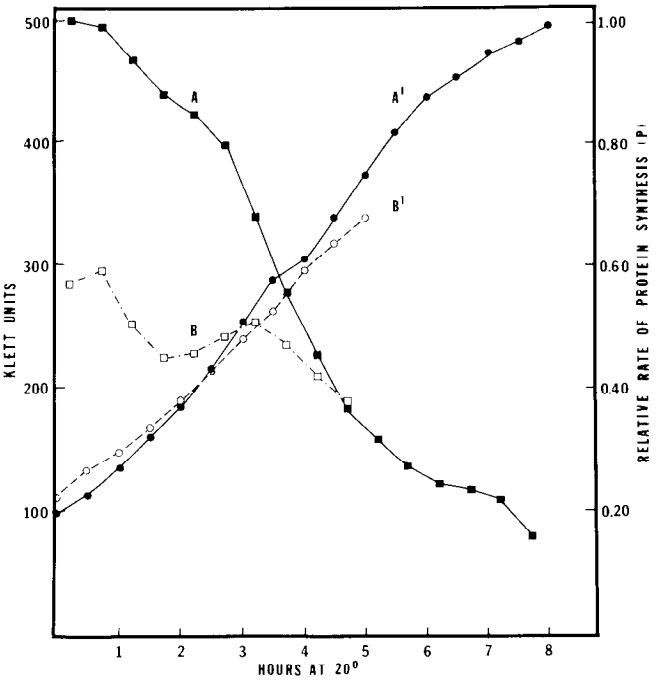

FIG. 2. Relative rates of protein synthesis and growth in *Bacillus megaterium* cultures at 20°. The curves show these parameters for the two cultures described in Fig. 1. These include protein synthesis (Curve *A*) and growth (Curve *A'*) for the culture grown from inoculum at 20° and protein synthesis (*B*) and growth (*B'*) for the culture transferred from 35° to 20° at 112 KU. Just before the shift from 35° to 20°, the transfer culture had a relative rate of protein synthesis of 1.05.

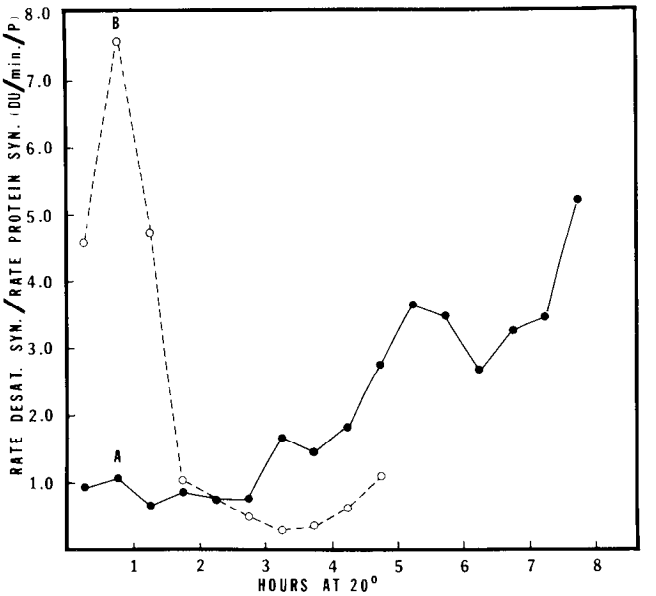

FIG. 3. Normalized rate of desaturase synthesis in *Bacillus megaterium* cultures at 20°. The curves illustrate this parameter for the cultures described in Fig. 1. Curve *A* was obtained for the culture growing from inoculum at 20° while Curve *B* was obtained for the culture transferred from 35° to 20°.

the normalized rate of desaturase synthesis were not carried beyond early stationary phase since, at this point, the decreasing rate of protein synthesis became too low to measure with accuracy.

For a culture transferred from 35° to 20°, the maximum normalized rate of desaturase synthesis (usually attained within the first hour after transfer) was always close to 8 DU/min/*P*. This was true despite significant variations, in some experiments, in the rates of general protein synthesis and of desaturase synthesis. Even when protein synthesis was severely depressed by the addition of inhibitors in amounts insufficient to cause total inhibition, the maximum normalized rate of desaturase synthesis remained approximately constant. This point is clearly illustrated in Table I. Finally, as *Curve B* in Fig. 3 suggests, the maximum normalized rate of desaturase synthesis is not attained immediately after transfer of a culture from 35° to 20° but is always preceded by a short lag period. The kinetics of hyperinduction during the first hour after transfer of a culture from 35° to 20° is more precisely defined in Table II. In this experiment, desaturase synthesis lags behind overall protein synthesis during the first 10 min after transfer, but by 10 to 15 min the normalized rate of desaturase synthesis has nearly attained its maximum value. It then remains at or near this value for the remainder of the first hour. As will be shown, however, the length of the period during which the normalized rate of desaturase synthesis is at or near its maximum can vary significantly with changes in culture density at the time of transfer.

Effects of Culture Density at Time of Transfer on Hyperinduction – When a *Bacillus megaterium* culture, growing at 35°, is transferred to 20°, the rate of growth after transfer is determined primarily by the culture density at the time of transfer. As expected, cultures grown to relatively high densities at 35° exhibited slower growth rates after transfer to 20° and entered the stationary phase sooner than comparable low density transfer cultures. Fig. 4 shows the levels of desaturase activity in *B. megaterium* cultures that were transferred to 20° after growing to various densities at 35°. Fig. 5 shows the rates of desaturase synthesis, normalized for protein synthesis, for each of these cultures. It can be seen (Fig. 5) that culture density or growth rate after transfer have little effect on the maximum normalized rate of desaturase synthesis during hyperinduction. With the exception of the culture transferred at 112 KU, the initial normalized rates of desaturase synthesis are also the same. However, culture density or growth rate (or both) does affect the later stages of the hyperinduction process. As Fig. 5 illustrates, the period during which maximum or near maximum normalized desaturase synthesis is maintained increases with increasing transfer density. Furthermore, even after repression of hyperinduction, significantly higher normalized rates of desaturase synthesis are maintained in the slower growing cultures. Finally, the rise in the normalized rate of desaturase synthesis usually associated with the beginning of stationary phase occurs sooner, as expected, in the higher density cultures.

Initiation of Hyperinduction in Culture Grown from Inoculum at 20° – Once the basic parameters of hyperinduction were established, we investigated the possibility that cultures growing from inoculum at 20° could be caused to hyperinduce desaturase by short incubation periods at 35°. As Fig. 6 shows, this was the case. When a culture growing at 20° is transferred to 35° for 30 min and then returned to 20°, a typical hyperinduction curve is obtained with desaturase synthesis peaking at 75 min (45 min after transfer back to 20°). A 20-min pulse at 35° also results in hyperinduction, but the maximum normalized rate of desaturase synthesis in this culture is only 3.75 DU/min/*P* compared to 6.65 DU/min/*P* for the culture pulsed for 30 min. A 10-min pulse at 35° causes only a low amplitude oscillation in the curve for the normalized rate of desaturase synthesis. Periods at 35° of less than 10 min were not attempted.

TABLE I

Effect of partial inhibition of protein synthesis on normalized rate of desaturase synthesis during hyperinduction

In the first experiment, a 1-liter culture was grown to a density of 188 KU at 35° and then divided into four equal portions. The first portion was retained as a control, while increasing amounts of chloramphenicol were added to the remaining three portions. All portions were then transferred to a 20.0° water bath and incubated with rapid stirring for 2 h. In the second experiment, a 1-liter culture was grown to 190 KU at 35° and then divided into three equal portions. These portions were then incubated for an additional 10 min at 35°, under conditions described below, and then transferred to a 20.0° water bath and incubated, with rapid stirring, for 3 h. The first portion was retained as a control, while nalidixic acid (10 μg/ml) was added to the second portion 10 min before transfer to 20.0° and to the third portion 10 min after transfer to 20.0°. In both experiments, desaturase synthesis and protein synthesis were determined in each portion at 30-min intervals throughout the 20° incubation period. Those values resulting in the maximum normalized rate of desaturase synthesis observed during the incubation are shown below.

Experiment and inhibitor	Amount of inhibitor added	Maximum normalized rate of desaturase synthesis	Rates of desaturase synthesis and protein synthesis at the maximum normalized rate	
			Desaturase	Protein
	μg/ml	*DU/min/P*	*DU/min*	*P*
1. Chloramphenicol	None	8.02	3.76	0.469
	10	8.20	2.00	0.244
	20	7.24	1.23	0.170
	40	8.97	0.78	0.087
2. Nalidixic acid	None	7.71	3.73	0.484
	10 (at −10 min)	6.67	1.32	0.198
	10 (at +10 min)	8.13	2.05	0.252

TABLE II

Kinetics of hyperinduction at 20°

A 1-liter culture of *Bacillus megaterium* that had grown at 35° ± 2° to a density of 230 KU was mixed rapidly with sufficient ice-cold medium to drop the temperature to 20° and then transferred at once to a 20.0° water bath shaker. The culture density immediately after dilution and transfer was 144 KU (zero time density) and was 187 KU after incubation at 20.0° for 60 min. Desaturase activity and protein synthesis were measured at regular intervals for 1 h after transfer, and from these data the normalized rate of desaturase synthesis during each interval was calculated.

Time interval after transfer to 20°	Desaturase synthesized during interval	Average rate of protein synthesis during interval	Normalized rate of desaturase synthesis during interval
min	*DU*	*P*	*DU/min/P*
0–5	0.6	0.235	0.51
5–10	3.1	0.274	2.26
10–15	10.6	0.279	7.60
15–20	11.6	0.282	8.23
20–25	9.0	0.284	6.34
25–30	8.8	0.287	6.13
30–45	30.2	0.297	6.78
45–60	33.7	0.318	7.06

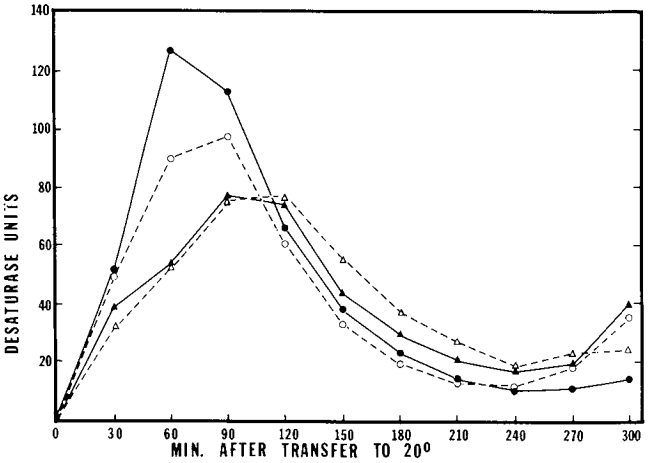

FIG. 4. Effects of culture density on desaturase activity during hyperinduction. Four 1-liter cultures of *Bacillus megaterium* were grown from inoculum at 35° in an incubator shaker and then transferred individually to a 20.0° water bath when growth reached specified cell densities. After transfer, incubation with rapid stirring at 20° was continued for 5 h. The level of desaturase activity and the rate of protein synthesis was determined in each culture at 30-min intervals. The cell densities in Klett units at the time of transfer and after 5 h at 20.0° for each culture were as follows: ●, 110 to 340; ○, 205 to 455; ▲, 320 to 490; △, 350 to 475.

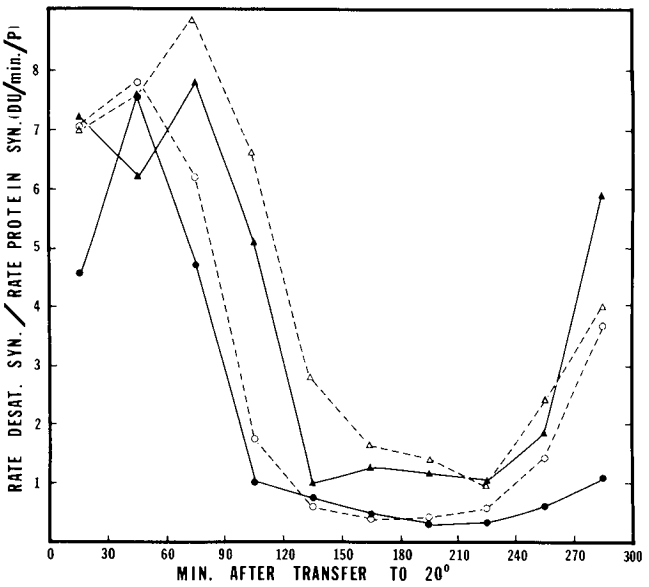

FIG. 5. Effects of culture density on the normalized rate of desaturase synthesis during hyperinduction. The curves illustrate this parameter for the cultures described in Fig. 4. The culture densities at the time of transfer from 35° to 20° were as follows: ●, 110; ○, 205; ▲, 320; △, 350.

When the culture, growing at 20°, was transferred to 35° for 1 h and then returned to 20° (results not shown in Fig. 6), essentially the same hyperinduction curve (maximum of 7.20 DU/min/*P* at 45 min after transfer back to 20°) was obtained as for the culture pulsed at 35° for 30 min.

Double Hyperinduction—The demonstration that desaturase hyperinduction could be initiated in cultures grown from inoculum at 20° by short periods of incubation at 35° prompted us to investigate the effect of 35° pulses on cultures that had already undergone hyperinduction by transfer from growth at 35° to incubation at 20°. Fig. 7 shows the results of an experiment in which a culture, after growth from inoculum at 35°,

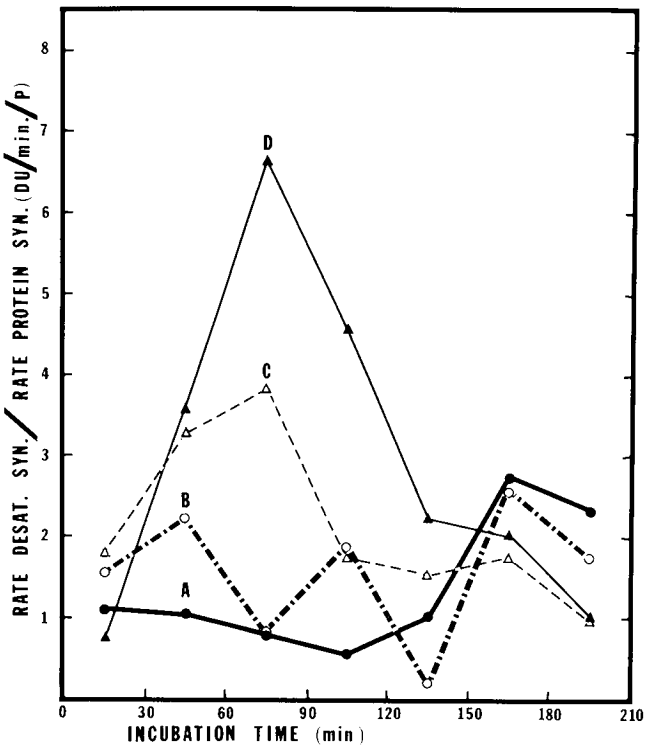

FIG. 6. Hyperinduction initiated in a culture growing at 20° by short term shifts to 35°. A 1-liter culture of *Bacillus megaterium* was grown from inoculum at 20° ± 1° in an incubator shaker. When the culture reached a density of 173 KU (zero time), it was divided into four equal portions. One portion (*A*) was transferred to a water bath at 20.0° and incubated with rapid stirring for 210 min. A second portion (*B*) was placed in a water bath at 35.0°, stirred at this temperature for 10 min, transferred to the 20.0° bath, and stirred rapidly for 200 min. Similarly, the third portion (*C*) and fourth portion (*D*) were stirred at 35.0° for 20 and 30 min, respectively, before transfer to the 20.0° bath. All initial transfers (from the incubator shaker to the water baths) were carried out within 1 min of zero time, and samples from each portion were taken at 30-min intervals thereafter to determine desaturation activity and protein synthesis rates. From these data the normalized rate of desaturase synthesis was calculated in the usual manner.

was transferred to 20°, incubated at the lower temperature for 60 min, and then pulsed at 35° for 30 min before being returned to 20°. A similar experiment, in which the pulse at 35° took place 90 min after the initial transfer is shown in Fig. 8. In both cases, there was a second hyperinduction peak of approximately equal magnitude to the first. In the latter experiment (Fig. 8) still a third hyperinduction peak, of lower amplitude than the first two, was obtained. When a culture, grown at 35°, was transferred to 20° for 90 min to cause hyperinduction and then pulsed for 30 min at 30° (rather than at 35°) before being shifted back to 20°, a second hyperinduction peak was not observed. Instead, a series of small amplitude peaks were obtained (Fig. 9) which were similar to those seen when a culture, growing from inoculum at 20°, was pulsed at 35° for 10 min (Fig. 6, *Curve B*).

Effect of Rifampicin on Hyperinduction—As Table III (Column A) shows, the RNA synthesis inhibitor, rifampicin (20, 21) totally blocked the induction of desaturase activity when it was added to a culture at the time of transfer from 35° to 20.0°. In this respect, rifampicin (25 μg/ml) was as effective as 100 μg/ml or more of chloramphenicol in preventing synthesis of desaturase, but unlike chloramphenicol, it did not immediately shut down protein synthesis. When rifampicin was

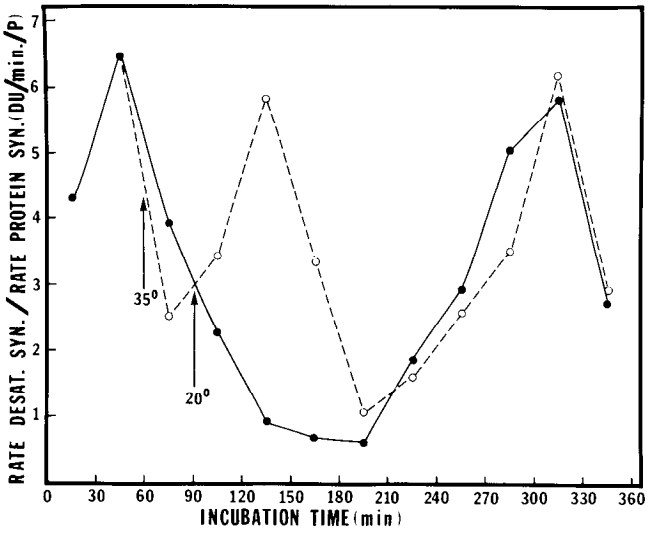

FIG. 7. Double hyperinduction. A culture of *Bacillus megaterium* was grown from inoculum at 35° ± 2° in an incubator shaker to 210 KU and then transferred (zero time) to a 20.0° water bath. Incubation, with rapid stirring, was continued for 60 min. At this time, one-half of the culture (now at 270 KU) was transferred to a 35.0° water bath and stirred rapidly at this temperature for 30 min before transfer back to 20.0°. The second (control) portion was maintained at 20.0° throughout the course of the experiment. Neither protein synthesis nor growth was affected significantly by the short period at 35°. Cell density after 360 min was 505 KU for the control culture and 510 KU for the double transfer culture. Desaturation and protein synthesis assays were carried out at 30-min intervals, and the normalized rate of desaturase synthesis was calculated for the control culture (●) and the double transfer culture (○).

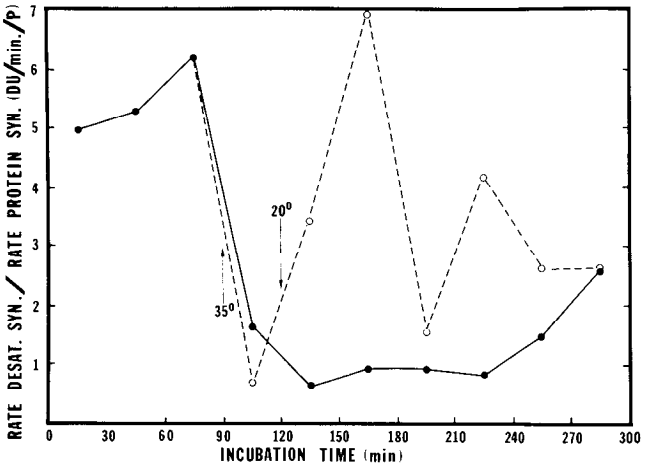

FIG. 8. Double hyperinduction. This experiment was carried out exactly as described in the legend for Fig. 7 except that the 35° pulse was applied at 90 min rather than at 60 min. Zero time culture density was 200 KU, and final (360 min) densities for the control and double transfer cultures were, respectively, 490 and 530 KU. The *curves* above show the normalized rate of desaturase synthesis for the control culture (●) and the double transfer culture (○).

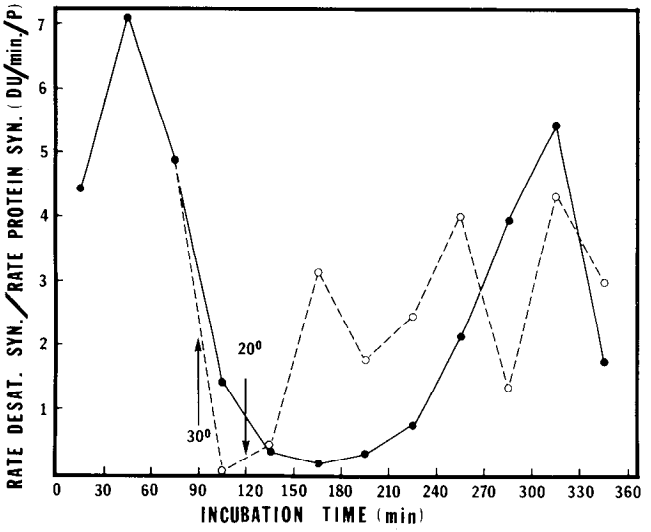

FIG. 9. Effect of transfer from 20° to 30° at 90 min. This experiment was carried out exactly as described in the legend for Fig. 7 except that one-half of the culture was transferred to 30° (rather than to 35°) at 90 min. Zero time culture density was 210 KU, density at 90 min was 290 KU, and final (360 min) densities for the control and double transfer cultures were, respectively, 490 and 480 KU. The *curves* above show the normalized rates of desaturase synthesis for the control (●) and double transfer (○) cultures.

TABLE III

Effect of rifampicin on rates of desaturase and protein synthesis in culture transferred from 35° to 20°

A 1-liter culture of *Bacillus megaterium* was grown from inoculum at 35° ± 2° to a density of 208 KU. It was then divided into three equal portions which were transferred at once to a 20.0° water bath. All portions were stirred rapidly at this temperature. Rifampicin (25 μg/ml) was added to the first portion (A) immediately after transfer and to the second portion (B) 40 min after transfer. The third portion (C), to which no rifampicin was added, served as a control. In addition to desaturase activity and protein synthesis, RNA synthesis was also estimated by measuring [2-^{14}C]uracil incorporation. However, there was essentially no uracil incorporation (less than 5% of the control) in portions A and B when measured within 1 min after the addition of rifampicin and no significant incorporation thereafter. Zero time samples for desaturation and protein synthesis were taken within 30 s after transfer of the cultures from 35° to 20.0°.

Time interval after transfer	Rate of desaturase synthesis			Rate of protein synthesis		
	A	B	C	A	B	C
min	*DU/min*			*P*		
0–15	0.00		2.48	0.131		0.480
15–30	0.00		2.57	0.026		0.304
30–45	0.00		3.25	0.008		0.339
45–60	0.00	0.66	2.89	0.005	0.125	0.365
60–75		0.48	1.64		0.047	0.368
75–90		0.08	1.41		0.029	0.354
90–120		0.12	0.82		0.017	0.351
120–150		0.00	0.57		0.006	0.359

added 40 min after the culture was transferred to 20.0°, desaturase activity decreased rapidly relative to that in the control culture, but, as Table III indicates, desaturase synthesis still proceeded at a significant (although decreasing) rate for the first 30 min after the inhibitor was added. Indeed, during this period, the normalized rate of desaturase synthesis in the inhibited culture (6.62 DU/ml/*P*) was about the same as in the control culture during the same period (6.18 DU/min/*P*).

Effect of Nalidixic Acid on Desaturase Synthesis in Transfer Cultures—The fact that the length of the hyperinduction period in cultures transferred from 35° to 20° increased with decreasing growth rate (Fig. 5) as well as the observation that the normalized rate of desaturase synthesis in cultures growing from inoculum at 20° increased as growth rate decreased (Figs. 1 to 3) suggested to us that the modulation of desaturase synthesis at 20° might somehow be associated with cell divi-

TABLE IV

Effect of nalidixic acid on rates of desaturase and protein synthesis in culture transferred from 35° to 20°

A 1-liter culture of *Bacillus megaterium* was grown to a density of 160 KU at 35° ± 2° and then divided into three equal portions. Nalidixic acid was added to Portions A (10 μg/ml) and B (25 μg/ml), while Portion C was retained as a control. All portions were then transferred to a 20.0° water bath and incubated with rapid stirring for 5 h. Desaturase activity and protein synthesis were determined at 30-min intervals during this period. The cell densities after 5 h for Portions A, B, and C were, respectively, 320 KU, 288 KU, and 420 KU.

Time after transfer	Absolute rate of desaturase synthesis			Rate of protein synthesis			Normalized rate of desaturase synthesis		
	A	B	C	A	B	C	A	B	C
min		*DU/min*			*P*			*DU/min/P*	
15	1.41	0.53	3.07	0.237	0.161	0.496	5.95	3.29	6.19
45	1.98	0.74	5.85	0.217	0.110	0.634	9.12	6.76	9.23
75	2.82	1.01	2.87	0.257	0.108	0.588	10.97	9.38	4.88
105	2.18	1.03	0.73	0.317	0.110	0.540	6.88	9.33	1.35
135	2.05	1.48	0.42	0.302	0.124	0.525	6.78	11.94	0.80
165	0.63	1.32	0.14	0.303	0.136	0.511	2.08	9.68	0.27
195	0.47	1.20	0.00	0.302	0.151	0.473	1.56	7.95	0.00
225	0.52	1.19	0.20	0.281	0.163	0.419	1.85	7.32	0.48
255	0.47	0.82	0.25	0.247	0.144	0.337	1.90	5.69	0.74
285	0.50	0.73	0.61	0.177	0.142	0.234	2.77	5.12	2.61

sion and DNA replication. To test this idea, nalidixic acid, an inhibitor of DNA synthesis (22–24) was added to a culture of *B. megaterium* just before transfer from 35° to 20°. As Table IV shows, 25 μg/ml of nalidixic acid did not markedly affect the normalized rate of desaturase synthesis during the hyperinduction phase but significantly stimulated the absolute as well as the normalized rate of desaturase synthesis during the period when these parameters were attenuated in the control. Unfortunately, the level of nalidixic acid (25 μg/ml) required to effect a 95% reduction in thymidine incorporation compared to a control also strongly inhibited both protein synthesis and RNA synthesis during the first 3 h after addition. Thus, from the experiment shown in Table IV, it was not clear whether nalidixic acid exerted its effect on desaturase synthesis by preventing DNA synthesis or whether its action could be attributed to one or more other inhibitory effects on macromolecule synthesis.

Effect of 6-(p-Hydroxyphenylazo)-uracil on Desaturase Synthesis in Transfer Cultures—To clarify the results obtained with nalidixic acid, a much more specific inhibitor of DNA synthesis, 6-(*p*-hydroxyphenylazo)-uracil, was tested for its effect on desaturase synthesis. This is the most specific known inhibitor of DNA synthesis in bacilli (25–28), and presumably acts by selectively inhibiting DNA polymerase III (29–33). We found that a concentration of 200 μM of this antibiotic quickly and totally inhibited DNA synthesis (as measured by thymidine incorporation) in *B. megaterium* but had little effect on either RNA or protein synthesis at this level. As Table V shows, 200 μM 6-(*p*-hydroxyphenylazo)-uracil also had very little effect on the rate of desaturase synthesis in a culture transferred from 35° to 20°.

Effect of Chloramphenicol on Desaturase Synthesis in Transfer Cultures—Chloramphenicol at a concentration of 100 μg/ml completely and quite specifically blocks protein synthesis in *B. megaterium* and stops all detectable desaturase synthesis almost immediately after addition (15, 17). Furthermore, we have found that 100 μg/ml of chloramphenicol does not significantly affect the rate of DNA synthesis at 20° for at least 3 h after addition. RNA synthesis remains normal for about 2 h after chloramphenicol addition but may drop rapidly relative to a control at later times.[1] To determine whether the effect of nalidixic acid on desaturase synthesis could be traced

[1] D. Fujii and A. Fulco, unpublished results.

TABLE V

Effect of 6-(p-hydroxyphenylazo)-uracil on rates of desaturase and protein synthesis in culture transferred from 35° to 20°

A 2-liter culture of *Bacillus megaterium* was grown at 35° ± 2° to a density of 183 KU and then divided into two portions. To the first portion (A) was added 6-(p-hydroxyphenylazo)-uracil (200 μmol/liter), while the second portion (B) served as a control. The portions were then transferred to a 20.0° water bath and incubated with rapid stirring for 4.5 h. Desaturase activity and protein synthesis were measured at 30-min intervals during 20.0° incubation. The cell densities after 4.5 h at 20° were 370 KU for Portion A and 405 KU for Portion B.

Time after transfer	Absolute rate of desaturase synthesis		Rate of protein synthesis		Normalized rate of desaturase synthesis	
	A	B	A	B	A	B
	DU/min		*P*		*DU/min/P*	
15	1.52	2.16	0.401	0.406	3.79	5.32
45	2.84	3.94	0.465	0.492	6.11	8.01
75	2.78	3.55	0.494	0.521	5.63	6.81
105	1.79	1.24	0.489	0.523	3.66	2.37
135	0.67	0.59	0.496	0.523	1.35	1.13
165	0.50	0.30	0.529	0.522	0.95	0.57
195	0.28	0.10	0.553	0.529	0.51	0.19
225	0.40	0.49	0.556	0.505	0.72	0.97
255	0.91	0.68	0.518	0.440	1.76	1.55

to its partial inhibition of protein synthesis, we examined the effect of chloramphenicol at concentrations which would strongly but not completely inhibit protein synthesis. We found that 40 μg/ml of chloramphenicol had about the same inhibitory effect on protein synthesis as 25 μg/ml of nalidixic acid. Table VI shows how concentrations of chloramphenicol below the levels necessary to completely inhibit protein synthesis affect desaturase synthesis in cultures transferred from 35° to 20°. Compared to the control (no chloramphenicol added), two concentrations of chloramphenicol sufficient to inhibit overall protein synthesis by about 60% and 80% had little effect on the normalized rate of desaturase synthesis during the hyperinduction phase but strongly stimulated normalized desaturase synthesis during the period when this parameter declined rapidly in the control. Indeed, at the higher chloramphenicol level, the absolute as well as the normalized rate of desaturase synthesis increased relative to the culture containing no chloramphenicol.

TABLE VI

Effect of chloramphenicol on rates of desaturase and protein synthesis in culture transferred from 35° to 20°

A 1-liter culture of *Bacillus megaterium* was grown to a density of 197 KU at 35° ± 2° and then divided into three portions. Chloramphenicol was added to Portions A (20 μg/ml) and B (40 μg/ml), while Portion C was retained as a control (no chloramphenicol added). All portions were then transferred to a 20.0° water bath and incubated with rapid stirring for 4.5 h. Desaturase activity and protein synthesis were determined at 30-min intervals during this period.

Time after transfer	Absolute rate of desaturase synthesis			Rate of protein synthesis			Normalized rate of desaturase synthesis		
	A	B	C	A	B	C	A	B	C
min		*DU/min*			*P*			*DU/min/P*	
15	0.98	0.86	2.16	0.249	0.127	0.504	3.93	6.77	4.29
45	1.32	0.74	2.47	0.217	0.114	0.549	6.08	6.49	4.50
75	0.84	0.67	3.90	0.191	0.093	0.510	4.40	7.20	7.64
105	0.77	0.53	0.82	0.167	0.074	0.449	4.61	7.16	1.83
135	1.03	0.47	0.47	0.144	0.084	0.409	7.15	5.60	1.15
165	0.30	0.66	0.19	0.142	0.092	0.400	2.10	7.17	0.48
195	0.34	0.63	0.25	0.160	0.090	0.381	2.13	7.00	0.66
225	0.33	0.52	0.28	0.168	0.091	0.358	1.96	5.71	0.78
255	0.59	0.34	0.78	0.183	0.087	0.275	3.22	3.91	2.84

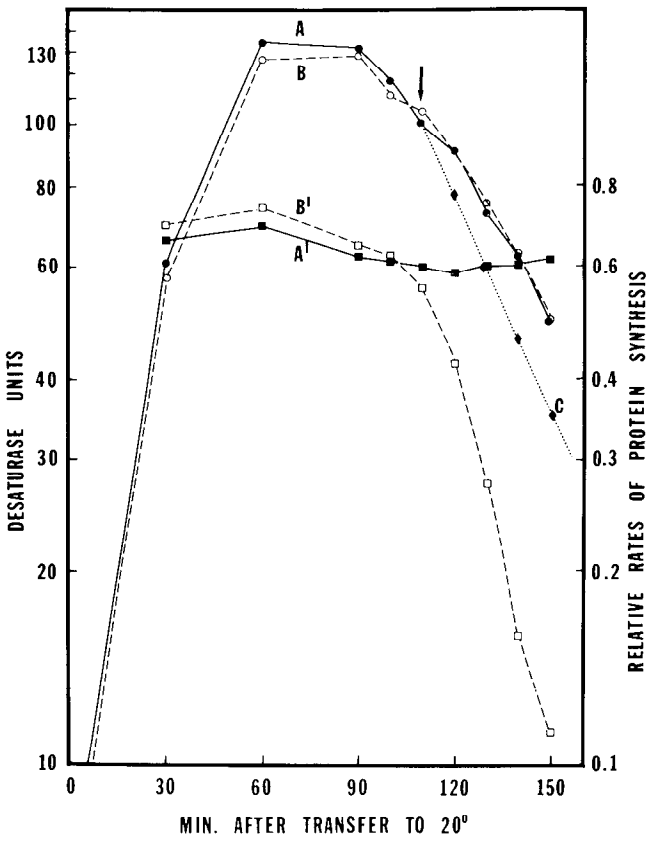

FIG. 10. Effect of rifampicin on attenuation of desaturase activity. A 1-liter culture was grown from inoculum at 35° ± 2° to a density of 205 KU. It was then divided into two equal portions which were transferred at once to a 20.0° water bath. Both portions were stirred rapidly at this temperature. The first portion (*A*) was retained as a control, while rifampicin (25 μg/ml) was added to the second portion (*B*) exactly 109 min after transfer to 20°. Desaturase activity (*Curves A* and *B*) and protein synthesis (*Curves A'* and *B'*) were measured at 30-min intervals during the first 90 min after culture transfer and at 10-min intervals thereafter. A third portion (*C*) was obtained by withdrawing a sample from Portion *A* at 109 min. Portion *C* was mixed immediately with chloramphenicol (100 μg/ml) and then stirred at 20.0° for 40 min. Desaturase activity (*Curve C*) was measured at 10-min intervals to determine the desaturase half-life. There was no detectable protein synthesis in Portion *C* after chloramphenicol addition. Note that the data for both desaturase activity and protein synthesis are plotted on a log scale.

A comparison of the data in Tables IV and VI shows that chloramphenicol, at concentration causing a similar level of protein synthesis inhibition, closely mimicked the effect of nalidixic acid on desaturase synthesis in cultures transferred from 35° to 20°.

Effect of Rifampicin on Attenuation of Desaturase Synthesis Following Hyperinduction – Fig. 10 shows the effect of rifampicin added to a culture 109 min after transfer from 35° to 20° on desaturase activity and protein synthesis. The rifampicin was purposely added at the time of maximum attenuation of desaturase synthesis (*i.e.* when the rate of desaturase synthesis in the control culture was decreasing most rapidly) and at a concentration that would completely and rapidly block RNA synthesis. It can be seen that under these conditions, rifampicin had no differential effect on desaturase synthesis. The decline in desaturase activity after 110 min in the control undergoing attenuation (*Curve A*) and in the rifampicin-treated culture (*Curve B*) were essentially the same, despite the inhibition of protein synthesis in the rifampicin-treated culture.

DISCUSSION

Before undertaking the work reported here, we developed a working hypothesis to account for certain previous observations (summarized briefly in the introduction to this paper) on the temperature-mediated control of desaturation in *B. megaterium*. In outline form, this hypothesis consisted of two basic tenets. First of all, we proposed that the inability of *B. megaterium* cultures, growing from inoculum at 35°, to synthesize desaturase enzyme could be ascribed to the absence, at this temperature, of the messenger RNA coding for its synthesis. The initiation of desaturase synthesis, then, after transfer of a culture from 35° to 20°, would require the prior synthesis of new messenger. Secondly, we suggested that the attenuation of hyperinduction at 20° as well as the relatively low rate of desaturase synthesis in cultures growing from inoculum at 20° could be explained by the action of a repressor or modulator at this temperature. It would follow that this modulator component was either totally absent or present at ineffective concentrations in cultures growing at 35°. Otherwise, one should observe simple desaturase induction (rather than hyperinduction) when a culture is transferred from 35° to 20°. The hyperinduction process, then, could be visualized as the unmodulated synthesis of desaturase initiated by the transfer of a culture from 35° to 20° with a consequent stabilization or rapid

derepression of desaturase messenger. At the same time, the synthesis of modulator would begin. Once modulator attained an effective concentration, it would then act to shut down hyperinduction and to attenuate the level of desaturase to that normally found in cultures growing from inoculum at 20°.

Although the results of the experiments reported in this paper are more complex than one could predict from the basic hypothesis, they are still consistent with the two major tenets of this hypothesis. Thus, the concept that desaturase messenger RNA is absent at 35° and hence must be induced after transfer of a culture to 20° is consistent with the observation (Table II) that there was a 10-min lag in desaturase synthesis relative to protein synthesis after culture transfer to the lower temperature. This initial lag period presumably represented the time necessary for the level of messenger coding for desaturase synthesis to build to maximal or normal concentrations. This interpretation is strongly supported by the results of an experiment (Table III) which showed that rifampicin, added at the time of transfer, completely blocked desaturase synthesis while permitting significant general protein synthesis to continue (at a decreasing rate) for almost 30 min. In contrast, the addition of rifampicin 40 min after culture transfer did not immediately shut down desaturase synthesis but rather caused its synthesis to decrease at a rate consistent with the decrease in the rate of general protein synthesis.

The postulate that there exists a modulator of desaturase synthesis (presumably a specific temperature-sensitive protein) that is absent at 35° but is produced at 20° is also supported by the experimental results. Of course, the very existence of the hyperinduction phenomenon implies that desaturase synthesis in a culture growing normally at 20° is controlled (modulated) in some way to maintain levels below those theoretically obtainable. We at first thought that the newly synthesized unsaturated fatty acids themselves, or else a change in membrane fluidity or structure brought about by their incorporation into membrane lipids, might somehow be responsible for the rapid decline in desaturase synthesis following hyperinduction, and for the relatively low level of desaturase synthesis in cultures growing from inoculum at 20°. However, we were forced to discard this idea when preliminary experiments[2] showed that the incorporation of exogenous monounsaturated fatty acids into the membrane lipids of *B. megaterium* at 35° had absolutely no effect on the kinetics of hyperinduction and subsequent decline of desaturase synthesis after culture transfer to 20°. Furthermore, as we shall show, the results reported in this paper also rule out the direct involvement of changes in unsaturated fatty acid composition in the control of desaturase synthesis.

Alternative explanations for modulation were subsequently considered, and we now feel that the logical choice, based on present evidence, is the synthesis of a temperature-sensitive modulator protein. The first test of this idea involved the transfer of a culture, growing from inoculum at 20°, to 35° for short intervals followed by return of the culture to 20°. We reasoned that if the modulator were a protein unstable at the higher temperature, then this manipulation should perturb or eliminate modulation. As Fig. 6 shows, this is exactly what happened when a culture, growing at 20° from inoculum, was given a 30-min pulse at 35°. The resulting hyperinduction curve was essentially identical with those observed when cultures growing at 35° were shifted to 20°. Pulses at 35° for 30 or more min were required for the complete (but transient) elimination of modulation. A 20-min pulse at 35° resulted in half-maximal hyperinduction, while a 10-min pulse caused only small cyclic perturbations in the curve for the normalized rate of desaturase synthesis. In cultures that had already undergone hyperinduction and attenuation after transfer from a growth temperature of 35° to a water bath at 20°, a second hyperinduction-attenuation cycle could be produced by a 30-min pulse at 35° (Figs. 7 and 8). This "double hyperinduction" effect is also consistent with the formation of a temperature-sensitive modulator at 20°. Finally, the finding that a 30-min pulse at 30° (Fig. 9) had an effect similar to that observed for a 10-min pulse at 35° (*i.e.* a low amplitude perturbation of modulation) suggests that modulator stability increases with decreasing temperature.

These results are also an additional argument against the possibility that alterations in the fatty acid composition of the bacterial membrane somehow mediate either hyperinduction or attenuation of desaturase synthesis. Although the fatty acid composition of *B. megaterium* varies widely with growth temperature (16, 17), a culture grown at 35° and transferred to 20°, a culture grown at 20° and pulsed at 35°, and a culture subjected to multiple temperature shifts (double hyperinduction) all gave essentially identical hyperinduction curves.

A consistent observation in all of the experiments reported here is that the maximal rate of desaturase synthesis achieved during hyperinduction was determined by the rate of protein synthesis during the same period, that is, the maximum normalized rate of desaturase synthesis was always a constant. One possible explanation for this observation is that this maximum normalized rate represents the rate of desaturase synthesis in the absence of the temperature-sensitive modulator. The validity of this explanation is supported by the results of a number of experiments which showed that various factors which alter the time of onset of attenuation as well as the degree of modulation, have no effect on the maximum normalized rate of desaturase synthesis achieved during hyperinduction. The demonstration that partial inhibition of protein synthesis by chloramphenicol or nalidixic acid does not affect that maximum normalized rate of desaturase synthesis (Table I) is additional evidence in support of this view. If, however, the plateau period of hyperinduction simply represents the unmodulated synthesis of desaturase, then we must explain why the onset of attenuation is delayed, after culture transfer from 35° to 20°, for periods up to several hours, depending on conditions, and why, after maximum attenuation, the normalized rate of desaturase synthesis increases again. A related question is why a culture, growing from inoculum at 20°, shows a gradual loss of modulation of desaturase synthesis during growth (Fig. 3).

We cannot yet answer these questions with certainty. We had previously suggested (18) that the kinetics of the modulation process could be understood, in part, if the DNA that codes for the modulator is not expressed at 35° and that, even after cultures are transferred to lower temperatures, DNA replication is required before desaturase synthesis is attenuated. We based this hypothesis on a number of good correlations between the effects of temperature and cell growth rate on one hand and on the modulation of desaturase synthesis on the other. The demonstration that nalidixic acid, which inhibits DNA synthesis, also eliminates or depresses modulation in hyperinduced cultures (Table IV) seemed to support this hypothesis. However, nalidixic acid, at the concentrations we found necessary to completely block DNA synthesis, had other effects as well, including partial inhibition of protein synthe-

[2] D. Fujii, unpublished results.

sis. Furthermore, as Table V shows, 6-(*p*-hydroxyphenylazo)-uracil, which strongly and quite specifically blocks DNA replication in bacilli by competitively inhibiting DNA polymerase III (29–33), has no effect on either hyperinduction or modulation.

Our failure to confirm the hypothesis that postulated a direct link between DNA replication and modulation led us to consider the possibility that nalidixic acid affected modulation of desaturase synthesis by partially inhibiting protein synthesis. To test this idea, we studied the kinetics of hyperinduction in transfer cultures that contained concentrations of chloramphenicol sufficient to cause partial inhibition of protein synthesis. A preliminary experiment (not shown) indicated that a chloramphenicol concentration of 10 μg/ml inhibited protein synthesis in a *B. megaterium* culture by an average of 35% during the first 4½ h after transfer from 35° to 20° but had little effect on the normalized rate of desaturase synthesis during this period, that is, there was no delay in the appearance of attenuation following hyperinduction nor was the degree of modulation significantly altered. However, as Table VI shows, at a chloramphenicol concentration of 40 μg/ml, the maximum normalized rate of desaturase synthesis was achieved as usual, but attenuation of desaturase synthesis was essentially eliminated. Indeed, from 135 to 225 min after culture transfer from 35° to 20°, the absolute (as well as the normalized) rate of desaturase synthesis in this culture was significantly greater than that in the control, despite a 78% average reduction in the rate of protein synthesis in the former. At 20 μg/ml of chloramphenicol (57% average inhibition of protein synthesis), modulation of desaturase synthesis was not eliminated but was partially blocked. This experiment (Table VI) provides additional evidence that desaturase synthesis is normally modulated at 20° and also demonstrates the dependence of modulation upon the rate of protein synthesis. In fact, modulation appears to be much more sensitive to partial inhibition of protein synthesis than is desaturase synthesis. This conclusion is consistent with the observation (Fig. 3) that the normalized rate of desaturase synthesis increases steadily, in a culture growing at 20° from inoculum, as the rate of protein synthesis decreases. It is also supported by the general finding that, after cultures have undergone hyperinduction and attenuation, the normalized rate of desaturase synthesis again rises as growth rate and the rate of protein synthesis decrease; indeed, when these cultures enter stationary phase, normalized desaturase synthesis may approach the maximum rate achieved during hyperinduction (see, for example, the curves for the control cultures in Figs. 7, 8, and 9). Finally, the idea that modulation and protein synthesis are coupled is supported by the observation (Fig. 5) that the onset of attenuation in hyperinduced cultures was delayed in proportion to culture density (since, in turn, the rate of protein synthesis was inversely proportional to culture density).

If we are correct in our conclusion that unmodulated desaturase synthesis is simply proportional to the rate of overall protein synthesis, then modulation appears to be an exponential function of the rate of protein synthesis. This is not to suggest, however, that the synthesis of the modulator protein itself is an exponential function of overall protein synthesis. Rather, one could speculate that the active modulator is an oligomer in equilibrium with an inactive monomeric precursor (26). The concentration of oligomer would then be an exponential function of the monomer concentration which in turn could be a simple function of overall protein synthesis. Such a relationship could explain why effective modulation of desaturase synthesis is delayed after a culture is transferred from 35° to 20° (thus permitting hyperinduction) but, once initiated, accelerates rapidly.

The mechanism by which modulator regulates desaturase synthesis remains unclear. The modulator does not seem to significantly affect the stability of the messenger RNA coding for desaturase synthesis. The rate of decrease of desaturase synthesis in the presence of rifampicin (taken as a measure of the messenger half-life) is about the same regardless of whether this inhibitor is added during maximum hyperinduction (Table III), presumably in the absence of modulator, or during maximum attenuation (Fig. 10) when modulator is present. The half-life of the desaturase messenger (9 to 11 min) calculated from these data is also consistent with the average half-life of the messenger RNAs coding for general protein synthesis (*i.e.* 10 to 15 min) in the presence of rifampicin. The experiment shown in Fig. 10 does suggest, however, that the modulator may act at the transcription level by shutting down the synthesis desaturase messenger RNA. The maximum rate of attenuation of desaturase synthesis in the control culture (*Curve A*, Fig. 10) corresponds closely with the rate of decay of desaturase synthesis caused by rifampicin addition (*Curve B*, Fig. 10).

One phenomenon that cannot be easily accommodated within our working hypothesis is the fluctuation in the normalized rate of desaturase synthesis in cultures growing at 20° from inoculum (Fig. 3) and the higher frequency and more uniform oscillations seen when cultures, at 20°, are subjected to a 10-min pulse at 35° (Fig. 6) or a 30-min pulse at 30° (Fig. 9). We have followed both cell growth and macromolecule synthesis in these situations and are satisfied that we were not dealing with synchronized or partially synchronized cultures. It would seem, therefore, that these oscillations in desaturase synthesis may indicate the presence of some feedback mechanism between the desaturase or one of its products and the modulator or some other component of the system that controls desaturase synthesis. Much more experimental work will be necessary, however, to establish (or disprove) such a relationship.

Acknowledgments – We thank Ellin James and Sue Tsuda for expert assistance in many of these experiments. We are also grateful to Dr. Harvey Herschman for a valuable discussion concerning this work and for suggesting the term "hyperinduction" to describe the major phenomenon reported here.

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D K Fujii and A J Fulco

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