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*PROPERTIES OF A TEMPERATURE-SENSITIVE REGULATORY SYSTEM**

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The synthesis of a number of inducible and repressible enzymes in bacteria has been shown to be controlled by the interaction of specific metabolites—inducers and "corepressors"—with specific, cytoplasmically diffused gene products which have been termed "aporepressors."[1] Little is known, however, about the nature of the postulated aporepressors, the molecular bases of their specificities, or the level(s) at which they intervene in the transcription of the information of structural genes.

In principle, some light might be shed on these questions through physiological experiments. Accordingly, numerous workers have examined the effect on enzyme repression of various regimes of unbalanced growth.[2] The ambiguities of these studies probably stem from two features of enzyme repression: (1) the synthesis of many inducible or repressible enzymes is subject to a complex and little understood repressive effect of carbon source dissimilation;[3] physiological manipulations which affect the pattern of catabolism consequently produce profound secondary effects on the rate of synthesis of regulated enzymes (e.g., the effect of thymine starvation on beta-galactosidase induction).[4] (2) If aporepressor molecules have a very high affinity for their sites of action[5] or a very high rate of synthesis,[6] then the sensitivity of the regulatory system may be too slight, or its response too transient, to be detectable. In particular, if aporepressors are rapidly synthesized and rapidly turned over, inhibitor studies would be complicated by the speed with which the steady state level of aporepressor will return to normal after the removal of inhibition.[6]

As a result of these considerations, we have sought a regulatory system in which the difficulties described above might be avoided. Such a system should: (1) be insensitive to catabolite repression, and (2) have a damaged regulatory system, hopefully one in which the rate of synthesis of aporepressor could be experimentally controlled. The synthesis of alkaline phosphatase in *E. coli* seems to satisfy condition (1), in that it is insensitive to catabolite repression[4, 7] while subject to a highly specific and genetically defined end product repression.[8] Consequently, we isolated a mutant which showed temperature-sensitive repression of alkaline phosphatase synthesis.[9] The present communication deals with the kinetics of phosphatase synthesis in this strain. Our observations indicate that the temperature sensitivity applies to the rate of synthesis of aporepressor. Variation in this rate

with temperature enables us to detect the fact that the aporepressor is metabolically unstable, with a time constant for inactivation during growth of about 0.2 generations, independent of temperature.

Methods and Materials.—Strain B3 is a thymineless mutant of *E. coli* B. PR1 is a 5-bromouracil induced mutant of B3 which shows temperature-dependent constitutivity of alkaline phosphatase synthesis[9] (Fig. 1). The medium employed in all experiments was Davis' minimal,[10] except that potassium phosphate was present in a concentration of 2×10^{-3} *M* (excess phosphate) or less, as specified, and Tris (0.05 *M*, pH 7.4) was employed as buffer. Thymine was always present at 10 μg per ml. Unless otherwise indicated, the carbon source was 0.2% glucose.

For enzyme assays, culture samples were centrifuged, resuspended in 0.1 *M* Tris, pH 7.4, and toluenized for 60–90 min at 37°. Alkaline phosphatase and beta-galactosidase were assayed according to Echols *et al.*,[8] except that the substrate concentration in the phosphatase assay was reduced to 1 mg/ml. Dihydroorotic acid dehydrogenase was assayed according to Yates and Pardee.[11] For each enzymatic activity, one unit corresponds to a change of optical density, at the appropriate wavelength, of 1.00 per min at 25°.

Cell density was measured as optical density at 720 mμ. Specific activities (and differential rates) are reported as enzyme activity divided by optical density at 720 mμ. An OD of 1.00 at 720 mμ corresponds to 220 ± 20 μg protein per ml under these conditions.

DNA, RNA, and protein were measured by conventional methods described elsewhere.[12]

Results and Discussion.—Figure 1 illustrates the temperature dependence of repression of PR1. The specific activity of alkaline phosphatase in limiting phosphate, as well as the specific activities of two control enzymes, is seen to be essentially temperature-independent. On the other hand, the specific activity of phosphatase in excess phosphate varies over a 300-fold range. At seven temperatures between 25° and 39°, we have verified that the specific activity observed after glucose exhaustion corresponds to a differential rate of synthesis during exponential growth. It should be noted that a limiting degree of repressibility is reached at temperatures below about 21°, the specific activity in excess phosphate dropping no lower than 0.002–0.003 units. This level is still somewhat above the specific activity of the fully repressed parental strain, which is 0.0005 ± 0.0002 independent of temperature.

The constitutivity of alkaline phosphatase synthesis at high temperatures is attributable either to an intracellular deficiency of corepressor, inorganic phosphate, or to a deficiency of aporepressor function. In PR1's repressible parent strain B3, derepression commences at growth-limiting concentrations of phosphate, as it does in strain K12.[7, 13] Therefore, an intracellular deficiency of phosphate sufficient to derepress alkaline phosphatase synthesis should also produce a decrease in growth rate. On the contrary, we find that in excess phosphate the growth rates of PR1 and of B3 are identical at 39° and 37°, as well as at 25°. A more critical test of phosphate uptake would be the dependence of growth rate on extracellular phosphate concentration. Consequently, we have compared in detail the growth of PR1 and B3 at phosphate concentrations low enough to be exhausted during growth. Such an experiment, which is shown in Figure 2, measures growth re-

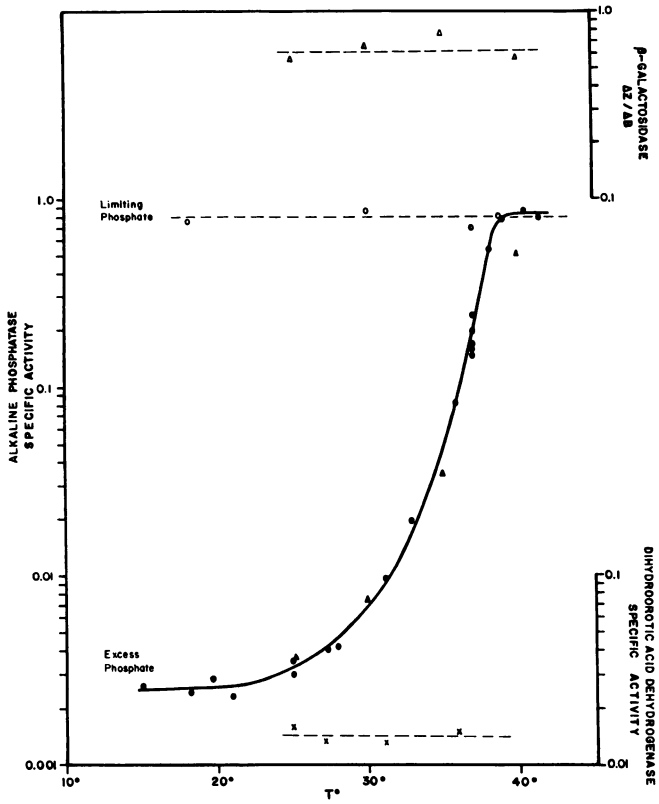

FIG. 1.—Temperature dependence of enzyme synthesis in PR1. Open circles: specific activity of alkaline phosphatase in overnight cultures grown in limiting phosphate (5×10^{-3} *M*). Filled circles: specific activity of alkaline phosphatase in cultures grown overnight in excess phosphate with limiting glucose (0.02%). Filled triangles: differential rate of alkaline phosphatase synthesis in excess phosphate with 0.4% glycerol. Open triangles: differential rate of induced synthesis of beta-galactosidase in excess phosphate, 0.4% glycerol, 10^{-3} *M* TMG. X's: specific activity of dihydroorotic acid dehydrogenase in cultures grown overnight in excess phosphate, limiting glucose.

sponse in a continuously decreasing concentration of phosphate.[7] Macromolecular synthesis responds in a characteristic way, as Horiuchi *et al.*[14] have described: a strikingly sharp break in the rate of increase of turbidity, an equally sharp break in RNA synthesis followed by RNA breakdown, and a more gradual decrease in the rate of protein and DNA synthesis. Figure 2 shows that the time course of these events is virtually identical in cultures of PR1 and B3 at 39°. It is highly implausible that at 39° PR1 maintains an internal phosphate concentration low enough to derepress alkaline phosphatase synthesis almost completely, yet high enough to maintain RNA, DNA, and protein synthesis at their maximum rates, and furthermore that it does so, independently of the extracellular phosphate concentration. We conclude that the temperature-sensitive variable in PR1 is the level of active aporepressor rather than the availability of corepressor.

The data of Figure 1 present the specific activity of alkaline phosphatase and its differential rate of synthesis under equilibrium conditions, i.e., after many generations of growth at each temperature. When a culture growing exponentially

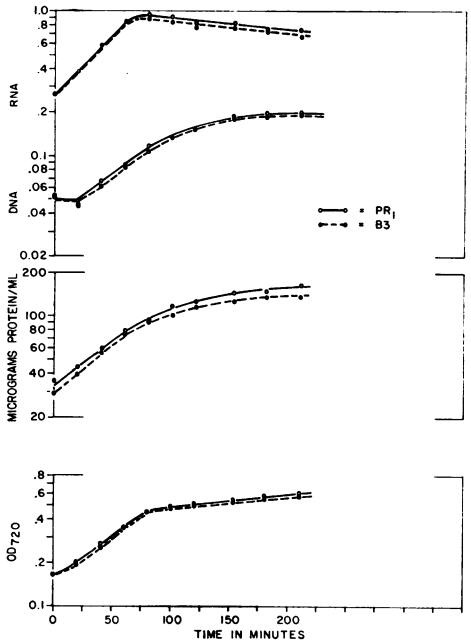

FIG. 2.—Growth of PR1 and B3 in 8×10^{-5} *M* phosphate at 39°. Cultures of B3 and PR1 growing in excess phosphate at 39° (differential rates of phosphatase synthesis were 0.0004 and 0.71, respectively) were harvested, washed in phosphate free medium, and cultivated at 39° in medium containing 8×10^{-5} *M* phosphate. DNA and RNA are shown on the same scale in arbitrary units. One arbitrary unit corresponds to an optical density of 1.00 at 260 mμ after hydrolysis in 0.5 *N* perchloric acid.

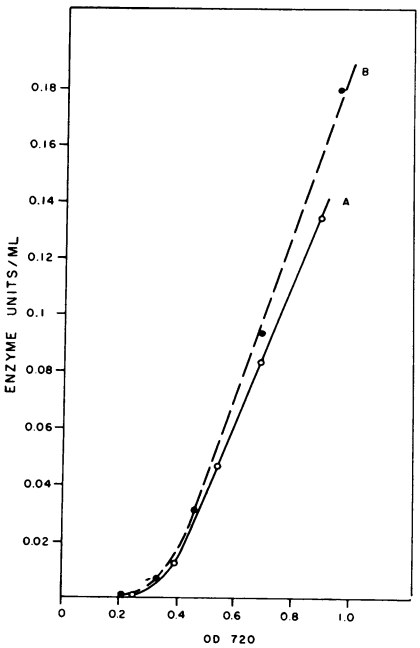

FIG. 3.—Alkaline phosphatase synthesis after temperature shift. A culture growing exponentially in excess phosphate at 25° was harvested, washed, and resuspended in the excess phosphate medium. Portion A was cultivated at 37° immediately. Portion B was incubated at 37° without glucose for 40 min (about the length of the lag period of enzyme synthesis in culture A) and then growth was initiated by the addition of glucose.

under conditions of repression, at 25°, is shifted to a higher temperature, the synthesis of enzyme responds as shown in Figure 3. The differential rate rises through a distinct lag period to a limiting value characteristic of the temperature. The lag period is too short to be accounted for by dilution of a stable, preformed aporepressor. Therefore, the progressive increase in differential rate during this period must reflect the inactivation of the material responsible for repression before the temperature was raised.

Comparison of curves *A* and *B* in Figure 3 reveals that this inactivation process is a matter of metabolic rather than of chemical instability. Preincubation at the higher temperature without growth (curve *B*) does not decrease the lag period or significantly affect the limiting differential rate subsequently attained. Thus, the inactivation is dependent on growth.

This point is amplified by the experiment illustrated in Figure 4. This experiment shows that, in a culture at temperature equilibrium, the differential rate of synthesis is not significantly affected either by heating in the absence of growth (curve *D*) or by decreasing the rate of growth at constant temperature (curves *B* and *C*).[15]

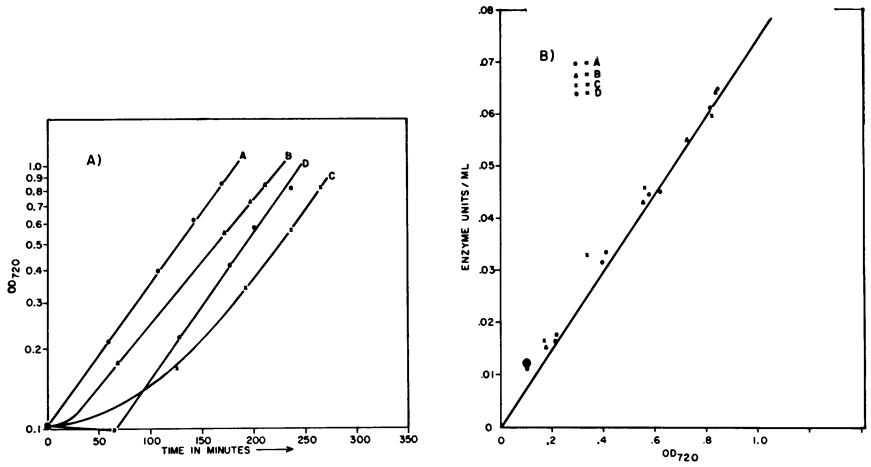

FIG. 4.—Effect of preheating and reduced growth rate. (In this experiment PR1A2, a leucineless derivative of PR1, was used, and leucine at 20 μg per ml was added to each culture.) A culture growing exponentially in excess phosphate at 35° was harvested, washed, resuspended in excess phosphate medium, and divided into four portions *A*, *B*, *C*, and *D*. *A* was cultivated at 35° on 0.2% glucose as a control. *B* was cultivated on 1% sodium lactate, producing a short lag and a modest (18%) decrease in growth rate. *C* was cultivated on 0.4% lactose, producing a long induction lag during which the growth rate was much lower than that of culture *A*. *D* was incubated without a carbon source (zero growth rate) at 37° for 60 min before return to 35° and addition of glucose to 0.2%. Fig. 4*A* presents the growth of these cultures as a function of time, while Fig. 4*B* shows their enzyme activity as a function of growth.

Two explanations for the growth-dependent inactivation of aporepressor are possible. On the one hand, it may be a process specifically originating from the mutational lesion in PR1. In this case, since repression in PR1 is temperature-dependent, the inactivation reaction should be temperature-dependent. On the other hand, the inactivation may represent a normal metabolic turnover of aporepressor. In this case the mutational lesion, and hence the temperature sensitivity, must apply to the rate of synthesis of aporepressor.

The simplest way to distinguish between these explanations is to determine the temperature dependence of the inactivation reaction. This was done in the experiment illustrated in Figure 5. Portions of a culture growing exponentially at 25° were transferred to 37° and 39° and sampled at frequent intervals throughout the lag period. Figure 6 presents the data of Figure 5 in a more informative way. Here we plot the average differential rate of synthesis during each successive growth interval versus the average OD during that growth interval. This plot shows that the initial rate of increase of differential rate of enzyme synthesis is not significantly different at the two temperatures. Therefore, the inactivation reaction is not temperature-sensitive.[19] Since the equilibrium differential rate is about threefold higher at 39° than at 37°, the temperature-dependent variable must be the rate of aporepressor synthesis.

It is worth noting that this argument is independent of the nature of the inactivation reaction, i.e., whether it were reversible or irreversible. If the temperature sensitivity applied to the inactivation reaction, we would have to assume that the extent of the reaction was temperature-dependent but that its rate was not. This hypothesis requires that the population of aporepressor molecules be heterogeneous,

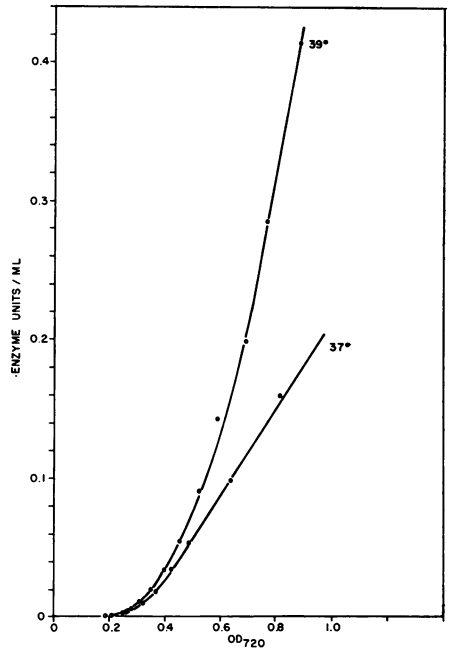

FIG. 5.—Alkaline phosphatase synthesis after temperature shift, two temperatures. A 25° culture was harvested as described above, and cultivated at 37° and 39°.

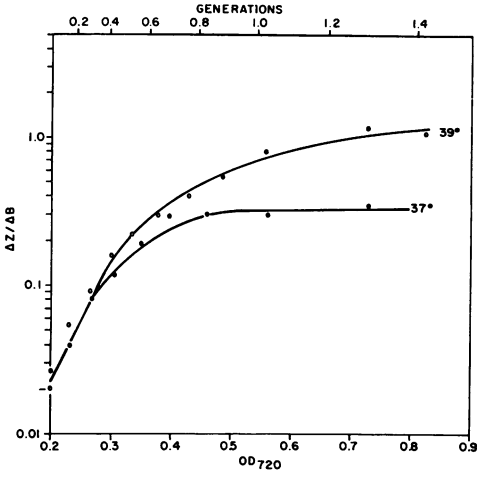

FIG. 6.—Rate of increase of differential rate of phosphatase synthesis. The data of Fig. 5 are replotted as described in the text. The upper abscissa gives generations of growth of each culture, where one generation is defined as the average doubling time divided by ln 2.

which is an *ad hoc* and highly artificial assumption. The heterogeneity of the population would have to be such that there were as many components, as a result of a single mutation, as there are points of differing specific activity in Figure 1.

On the contrary, if the rate of aporepressor synthesis is inversely temperature-dependent, then each point in Figure 1 simply represents a different steady state level fixed by the constant rate of inactivation and a different rate of synthesis. An inverse temperature dependence in rate of synthesis is entirely plausible if an enzyme concerned with aporepressor formation has been rendered temperature-sensitive by the mutation. Garen and Echols[16] have suggested, on the basis of elegant genetic experiments, that the R2 regulator gene of strain K12 specifies the structure of an enzyme which converts an internal inducer of alkaline phosphatase synthesis into a repressor. Crosses of PR1 with appropriately marked donor strains confirm that the constitutive mutation in PR1 is at the R2 locus (Table 1).

It is likely that the synthesis of aporepressor represents a function distinct from its growth-dependent inactivation. Thus, the inactivation documented in Figure 6 probably reflects a metabolic turnover which occurs in repressible strains but which is masked by the high and invariant rate of synthesis. An initial e-fold increase in differential rate occurs in approximately 0.2 generations (Fig. 6), which suggests a time constant for aporepressor turnover of this order of magnitude. This estimate is rough, of course, since we have no independent information on the order of the reaction or on the relationship between aporepressor concentration and differential rate of enzyme synthesis.

TABLE 1

MAPPING OF PR1

Recipient	Donor	Recombinant class screened	Colonies screened (no.)	Repressed colonies (no.)	Repressed colonies (%)
PR1LS	W 2252	Sm^rLeu^+	571	14	2.5
$Leu^-Mal^-Sm^rVal^r$	$R1^+R2^+Leu^+Sm^sVal^sMal^+$				
		Mal^+Val^r	44	3	7.0
PR1LS	C 8	Sm^rLeu^+	184	6	3.0
	$R1^-R2^+Leu^+Sm^sVal^sMal^+$				
		Mal^+Val^r	1697	111	6.5
PR1LS	C 97	Sm^rLeu^+	2584	4	0.15
	$R1^+R2^-Leu^+Sm^sVal^sMal^+$				
		Mal^+Val^r	994	1	0.1

The recipient strain PR1LS is a streptomycin-resistant leucine requiring derivative of PR1. Like all our strains of *E. coli* B, it is resistant to valine inhibition. The donor strains are derivatives of *Hfr Cavalli* which are sensitive to valine. C8 and C97 were generously supplied by Dr. Allan Garen. Log phase donors were shaken gently in Pennassay broth at 37° with an excess of recipients. After 120 min the cultures were diluted 20-fold in tryptone broth and cultivated at 37° for 120 min. They were then harvested, washed, and plated. Recombinants were selected on tris minimal-excess phosphate medium supplemented with 10 μg per ml thymine and 20 μg per ml methionine. For Sm^rLeu^+ recombinants the carbon source was 0.2% glucose, and streptomycin was present at 30 per ml. For Mal^+Val^r recombinants the carbon source was 0.4% maltose, leucine was present at 20 μg per ml, and valine at 400 μg per ml. The plates were incubated at 37°. Alkaline phosphatase production was determined by spraying the recombinant colonies with p-nitrophenylphosphate at 15 mg/ml in 1 *M* tris, pH 8.0.

We suggested in the introduction that a steady state situation of this sort would frustrate physiological studies on enzyme repression because of the rapidity with which the aporepressor level would return to equilibrium after it had been disturbed. If our analysis is correct, PR1 should have the distinctive and useful property of returning more slowly to equilibrium, the higher the temperature. Experiments showing that this is precisely the case are reported elsewhere.[17] This property has enabled us to carry out inhibitor studies on PR1, under the hoped-for conditions of slow aporepressor synthesis, which provide circumstantial evidence as to the nature of the aporepressor.[18]

Summary.—The synthesis of alkaline phosphatase has been examined in a mutant of *E. coli* B in which repression by inorganic phosphate decreases with temperature. It is concluded that the temperature sensitivity lies in the rate of synthesis of an aporepressor which is normally metabolically unstable. The time constant for aporepressor turnover is estimated to be about 0.2 generations.

* This work was partially supported by grant NG-9546 from the National Institutes of Health.

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[19] By "temperature-sensitive," we mean responding differently to temperature than growth rate. The use of the differential plot automatically normalizes to the growth rate.

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*A MOLECULAR MECHANISM OF MITOMYCIN ACTION: LINKING OF COMPLEMENTARY DNA STRANDS**,†

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The selective action of the antibiotic mitomycin C (MC)[1] on deoxyribonucleic acid (DNA),[2-6] together with its reported antineoplastic,[1, 7] mutagenic,[8] and phage-inducing[9] activities, has stimulated several investigations on the mechanism of its action. The preferential inhibition of bacterial DNA synthesis by MC, accompanied by progressive and extensive breakdown of the DNA, indicates that DNA is the principal target. However, the rapidity of MC-induced "death" seemed to be out of step with the relatively much slower process of DNA breakdown. This suggested that the effects hitherto observed might be secondary to an earlier action of the antibiotic on DNA. Such a primary lesion is described here and interpreted as *in vivo* MC-induced linking ("cross-linking") of the complementary strands of the DNA molecule.

Materials and Methods.—Samples of mitomycin C were kindly provided by Dr. J. Lein, Bristol Laboratories, Syracuse, N. Y., by Dr. R. B. Ross, Cancer Chemotherapy National Service Center, NIH, Bethesda, Md., and by the Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. The bacterial strains used included *Escherichia coli* strain B, *Sarcina lutea* strain ATCC-272, and the following *Bacillus subtilis* mutant lines:[10, 11] wild-type, the indole-requiring, the linked indole- and histidine-deficient (I^-, H^-), and a prototrophic derivative of the indole-requiring strain 168, the latter bearing in addition a marker (*mac-r*1) conferring resistance to the macrolide group of antibiotics. Bacteria which were growing exponentially in Difco's antibiotic medium 3 (Penassay broth) were exposed to MC under the conditions specified, and their survival determined by plating on nutrient agar. To terminate MC exposure the cells were chilled, washed twice with cold SSC (0.15 *M* NaCl + 0.015 *M* Na_3·citrate), and frozen with 100 μg lysozyme/ml. The procedures used for the isolation of protein- and RNA-free DNA and for the determination of its buoyant density and transforming activity were outlined earlier.[11] Thermal transition ("melting") curves were obtained with a recording thermospectrophotometer.[12] Thermal denaturation was carried out by exposing the DNA in 0.015 *M* NaCl + 0.0015 *M* trisodium citrate at pH 7.7 (DSC) to 100°C (or other specified temperatures) for 6 min followed by rapid cooling in an ice bath.[11]

Results.—The exposure of an exponentially growing culture of *B. subtilis* to an inhibitory concentration of MC resulted in very rapid cell death, colony-forming capacity dropping by several powers of ten in a matter of minutes (Fig. 1, Table 1). Native DNA extracted at this early period from MC-exposed cells and examined