JOURNAL OF BACTERIOLOGY, Apr. 1968, p. 1450-1460
Copyright © 1968 American Society for Microbiology

Vol. 95, No. 4
Printed in U.S.A.

Deoxyribonucleic Acid Degradation in *Bacillus subtilis* During Exposure to Actinomycin D[1]

JAMES L. FARMER

Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island, and Department of Biophysics, University of Colorado Medical Center, Denver, Colorado 80220[2]

Received for publication 15 December 1967

At high concentrations (10 μg/ml), actinomycin D inhibited deoxyribonucleic acid (DNA) synthesis in *Bacillus subtilis*. Inhibition occurred quickly (in less than 1 min) and was complete. In strain 23 *thy his*, inhibition of DNA synthesis by actinomycin D was followed by partial degradation of one of the two daughter strands to acid-soluble products. Degradation began at the replication point and proceeded over a distance equal to about 12% of a chromosome in length. Actinomycin D played some essential part in degradation, since exposure of the cells to other treatments or agents which inhibit growth did not lead to the above result.

Actinomycin D (ACT) is a naturally occurring antibiotic which at low concentrations specifically inhibits deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) synthesis. At high concentrations, ACT inhibits DNA replication as well (6, 13, 25, 28, 30). In vitro inhibition of DNA synthesis is not due to competition between synthesis and destruction by ACT, but is in fact inhibition of new synthesis (6). Inhibition of DNA synthesis by ACT is due to alteration of the DNA primer rather than to direct interference with DNA polymerase (14, 25). Concentrations of ACT which inhibit DNA synthesis also cause a marked increase in the temperature required to denature the DNA (17, 25). This and other experiments have been interpreted to mean that bound ACT increases the binding energy of the guanine-cytosine pair in DNA (17), thus stabilizing the β form of the DNA (12) and preventing the strands from separating.

There are two classes of DNA-binding sites for ACT (2, 10). Although there is some disagreement, it appears that there is approximately one strong binding site per 14 nucleotides in most natural DNA molecules; the number of weak binding sites is approximately equal to the number of guanine residues (10). Binding at the strong sites is responsible for inhibition of RNA synthesis, whereas binding at the weak sites is almost certainly responsible for the physical changes in DNA which make it unsuitable as a primer for DNA synthesis. Both classes of sites must involve guanine residues, since artificial DNA which contains only adenine and thymine does not bind ACT (11), and since there is an absolute requirement for the 2-amino group of purines for binding to take place (3). Denaturation of DNA leads to loss of both classes of binding sites and their replacement by a new class which is intermediate in its properties (2). The exact nature of either type of binding site is not clear, but the model of Hamilton et al. (12) seems to be in good agreement with the known properties of the strong binding sites. It seems unlikely that the weakly bound ACT could be attached by intercalation (16) because it is inclined 23° to the plane of the DNA bases (10), and because it does not increase either the viscosity of the DNA (20) or its radius of gyration (2).

There is steric hindrance between ACT molecules bound to two guanine residues which are close to one another (10, 11). The binding energy of the ACT-DNA complex is probably derived from hydrophobic interactions (10, 21, 22).

Concentrations of ACT which are sufficient to inhibit DNA synthesis also protect DNA from digestion by deoxyribonucleases in vitro (7, 29). These concentrations also inhibit dark repair of ultraviolet (UV) damage in *Bacillus subtilis* by preventing breakdown of DNA which contains photoproducts (27).

This paper will describe ACT-induced degradation of a daughter strand adjacent to the DNA replication point in a strain of *B. subtilis* during exposure to a concentration of ACT which was sufficient to inhibit DNA synthesis.

[1] From the Eleanor Roosevelt Institute for Cancer Research and the Department of Biophysics (Contribution No. 316), University of Colorado Medical Center, Denver.

[2] Present address.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strains 23 *thy* and 168 *thy ind* have been described elsewhere (9). Strain 23 *thy his* is a histidine-requiring strain which was derived from strain 23 *thy* by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine followed by enrichment for amino acid auxotrophs by thymine starvation.

Media. Cells were grown in a medium composed of Spizizen salts (1), 0.5% glucose, 1.0% monosodium glutamate, 20 μg (per ml) of L-histidine, and 5 μg (per ml) of thymine (SGGHT). The doubling time of strain 23 *thy his* in this medium at 37 C was 47 $\pm$ 4 min. Viable count was determined by dilution into SGGHT and plating with melted (45 C) SGGHT plus 1.5% Difco Noble Agar.

Isotopes. Thymine-*2*-^{14}C and thymine-methyl-^{3}H were purchased from the New England Nuclear Corp. ACT-methyl-^{14}C was a gift from the same corporation.

Some batches of the commerical radioactive thymine were checked for uracil contamination by paper chromatography in two solvents (65 ml of isopropanol, 17.6 ml of concentrated HCl, 17.4 ml of water; and water-saturated *n*-butanol in an ammonia atmosphere). Only one peak was obtained in all cases, that corresponding to thymine.

Antibiotics. ACT (lot numbers L-554,651-0-6, L-554,651-0-7, and L-554,651-0-10) was a gift from the Merck Sharpe and Dohme Research Laboratory. It was stored at 4 C in 70% ethyl alcohol. Phleomycin (lot number A9331-648) was a gift from Bristol Laboratories. It was stored at 4 C in aqueous solution. Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 9-aminoacridine were purchased from the Aldrich Chemical Co.

Isotopic labeling of DNA. Cells were labeled in one of two ways: thymine-methyl-^{3}H was added directly to a cell suspension; or a given volume of cell suspension was transferred to an equal volume of prewarmed and aerated medium which was identical in composition except for replacement of thymine by thymine-*2*-^{14}C or thymine-methyl-^{3}H. Pulse labels were terminated by filtering the cells (Millipore Corp.; HAWP047) and washing them with several volumes of fresh medium. (Wash time from filtration to resuspension was 4 to 5 min.) Unless otherwise indicated, cells were labeled with thymine-*2*-^{14}C.

Treatment of cells with ACT. Unless otherwise stated, cells were exposed to 10 μg of ACT per ml (one part of stock solution to 100 parts of cell suspension). Control cultures received an equivalent amount of ethyl alcohol. Cultures were incubated in dim light after addition of ACT. Unless otherwise indicated, the final cell concentration was between 2×10^7 and 3×10^7 per ml.

Estimation of trichloroacetic acid-precipitable counts in whole cells. At intervals, 0.5 ml of cell suspension was precipitated with 0.5 ml of cold 10% trichloroacetic acid. After several hours in the cold, the precipitates were collected on Millipore membranes (HAWG-023) and washed with cold 5% acid. The dried filters were counted in a low-background, gas-flow counter or were placed in a toluene-base scintillation fluid and counted in a scintillation counter. Low-activity samples were counted until at least 500 counts had accumulated.

Adding unlabeled thymine or ACT to the acid did not significantly decrease the number of acid-precipitable counts obtained by the above procedure. Thymine-*2*-^{14}C was added to a precipitate of unlabeled cells in cold 5% acid. The sample was divided into three fractions, one of which was untreated, the other two of which were supplemented with either thymine (20 μg/ml) or ACT (5 μg/ml). After several hours in the cold, the samples were collected and washed as described above. The percentage of labeled thymine recovered in the acid-precipitable fraction was: untreated, 0.11; thymine-treated, 0.089; ACT-treated, 0.070.

Cesium chloride gradients. Cells were lysed as follows. Samples (5 ml; approximately 7×10^7 cells) were chilled, supplemented with 0.02% Merthiolate, and concentrated into 0.9 ml of standard saline-citrate (SSC; 23); they were incubated with lysozyme (1 mg/ml) at 37 C for 30 min, chilled in ice, and treated with 0.1% sodium dodecyl sulfate for 10 min; the cells were then frozen (dry ice) and thawed (37 C) three times and incubated with Pronase (1 mg/ml) overnight at 37 C. (Pronase was incubated at 37 C in aqueous solution for a few minutes before it was added to the cell lysate, in order to inactivate any contaminating nucleases.) The volume was adjusted to 2.1 ml with SSC, 2.8 g of CsCl was added, and the solution was centrifuged in a Spinco SW-50 rotor at $100{,}000 \times g$ and 25 C for 48 hr. Fractions were collected from the bottom of each tube, precipitated with cold 5% trichloroacetic acid, and assayed for radioactive counts in a scintillation counter, as described above.

RESULTS

Inhibition of DNA synthesis by ACT. At low concentrations, ACT did not seem to affect DNA synthesis directly, but at high concentrations (10 μg/ml) it completely inhibited DNA synthesis in less than 1 min (Fig. 1). The magnitude of inhibition at each concentration is in good agreement with the values measured in in vitro experiments (13, 25). Although the data shown are for strain 23 *thy his*, strain 168 *thy ind* gave identical results in a similar experiment.

Degradation of DNA during exposure of cells to ACT. When cells which contained label only in the parental DNA strands (long or short regions) were exposed to ACT, a fraction of the label was degraded to acid-soluble material (Fig. 2). The extent of loss was small (11% in 140 min) and the rate of loss was essentially constant over the period observed. This loss, which will be referred to as "nonspecific degradation," may have been due to autolysis of dead cells.

The pattern of degradation was quite different in cells which had been pulse-labeled immediately

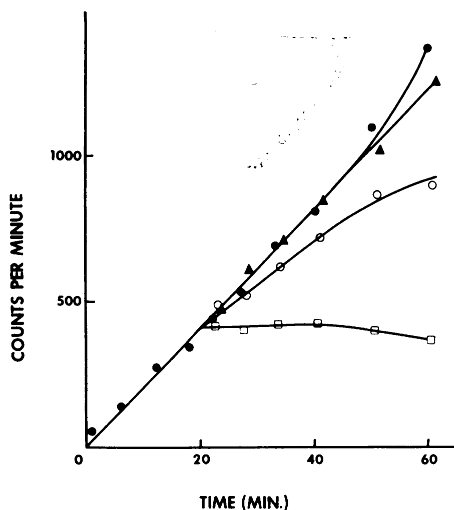

FIG. 1. *Inhibition of DNA synthesis by ACT. An exponentially growing culture of strain 23 thy his in SGGHT was diluted into the same medium supplemented with thymine-2-^{14}C at time zero. At 20 min, the culture was divided into four parts and supplemented with ACT (□, 10 μg/ml; ○, 2 μg/ml; ▲, 0.5 μg/ml; ●, control).*

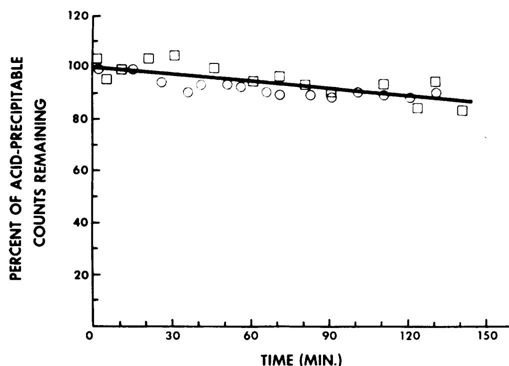

FIG. 2. *Degradation of DNA after addition of ACT to pulse-labeled cells (parental strands). Exponentially growing cultures of strain 23 thy his were labeled, grown for some time in unlabeled medium (chased), treated with ACT, and assayed for acid-precipitable counts. Control cultures were taken as 100% (not shown) and the experimental cultures were then normalized to the control cultures. (○) label = 24 doubling times, chase = 3 doubling times, 100% = 1,740 counts/min; (□) label = 0.12 doubling time, chase = 1.0 doubling time, 100% = 183 counts/min.*

before exposure to ACT (Fig. 3). There was an initially rapid rate of loss which approached zero after 60 min. The rate and extent of loss were dependent on the size of the labeled region, each being larger for short pulses than for long.

Dose dependence of degradation. The minimum concentration of ACT which led to the maximal degradation of pulse-labeled DNA was 10 μg/ml. Higher concentrations did not increase the fraction of counts lost from pulse-labeled cells, although they did increase the magnitude of nonspecific degradation (Table 1).

Is the degraded material really DNA? When cells were pulse-labeled with radioactive thymine (^{3}H or ^{14}C), essentially all of the initially present acid-precipitable counts banded with DNA in a CsCl density gradient, as did the counts remaining after exposure to ACT (Fig. 4). This result indicates that nearly all of the acid-precipitable counts found in cells after short pulses were incorporated into DNA rather than into substances other than DNA. Further evidence that the observed degradation is not an artifact is that degradation did not occur in strain 168 *thy ind* (Table 2). DNA synthesis in the latter strain showed precisely the same sensitivity to ACT as did strain 23 *thy his*, and nonspecific degradation did occur in strain 168 *thy ind* (12% of the initially acid-precipitable counts were degraded in 140 min). Strain 168 *thy ind* lacks the degradative mechanism which is operative in strain 23 *thy his.*

Degradation not affected by absence of his *marker.* Since the *his* marker was introduced into strain 23 *thy* by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a very potent mutagen, the possibility existed that degradation was due to introduction of a second mutation along with the *his* mutation, or perhaps was due to the presence

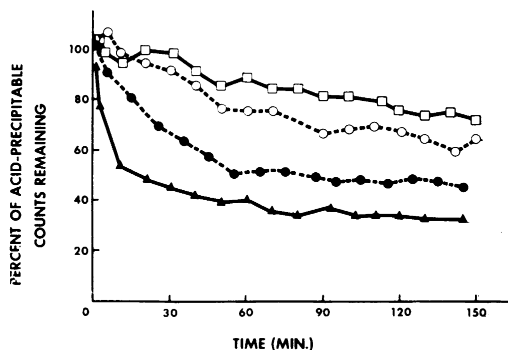

FIG. 3. *Degradation of DNA after addition of ACT to pulse-labeled cells (daughter strands). Exponentially growing cultures of strain 23 thy his were labeled, treated with ACT, and assayed for acid-precipitable counts. Control cultures were taken as 100% (not shown), and the experimental cultures were then normalized to the control cultures. (□) Label = 0.31 doubling time, 100% = 557 counts/min; (○) 0.19 doubling time, 100% = 502 counts/min; (●) 0.097 doubling time, 100% = 424 counts/min; (▲) 0.024 doubling time, 100% = 83 counts/min.*

TABLE 1. *Influence of ACT concentration on degradation of DNA*[a]

ACT	A (mean ± SD)	B (mean ± SD)
μg/ml		
0	1.00 ± 0.014[b]	1.00 ± 0.025
1	0.99 ± 0.028[b]	0.92 ± 0.023
5	0.93 ± 0.076	0.80 ± 0.006
10	0.54 ± 0.044	0.65 ± 0.040[c]
20	0.47 ± 0.026	0.60 ± 0.007
50	0.49 ± 0.035	0.89 ± 0.006

[a] Cultures were labeled with thymine-*2-¹⁴C* as described below. Samples of each culture were shaken with various concentrations of ACT for 2 hr at 37 C, and acid-precipitable counts were measured. In column A, the cells were labeled for a period corresponding to 0.12 doubling time, after which the label was removed. In column B, the cells were labeled for a period corresponding to 4 doubling times, after which the label was removed and the cells were allowed to grow in unlabeled medium for a period corresponding to 2.6 doubling times. In both A and B, the results are expressed as counts per minute divided by the control counts per minute. (A control, 55 counts/min; B control, 838 counts/min.)

[b] Average of two samples. All others, average of four samples.

[c] In this experiment, nonspecific degradation was greater than 11% at a concentration of 10 μg/ml. This result was consistent in experiments in which cells were shaken during incubation with ACT, whereas 11% was the consistent result in experiments in which the cells were aerated by bubbling.

of the *his* marker itself. The parent strain, 23 *thy*, was pulse-labeled with thymine-*2-¹⁴C* and exposed to ACT. It behaved exactly like strain 23 *thy his* (Table 2).

Viability of B. subtilis in medium containing ACT. At low concentrations (e.g., 0.5 μg/ml), ACT was bacteriostatic and dilution alone was sufficient to allow exposed cells of strain 23 *thy his* to form colonies. At higher concentrations (e.g., 10 μg/ml), ACT was bactericidal (Fig. 5). The death curve for strain 23 *thy his* is biphasic, the initial half-life being 318 ± 100 (standard deviation) min. The curve breaks at 90 ± 12 min, the new half-life being 46 ± 3.3 min. The data in the text are the average of four experiments. Strain 168 *thy ind* is much more sensitive to ACT (Farmer, *unpublished data*).

Models of degradation. There are five possible explanations for the observed degradation. (A) Random degradation of the DNA molecules. This can be immediately dismissed since it does not explain variation in the fraction of counts lost as a function of the size and position of the labeled region of the DNA. This model may, however, account for nonspecific degradation. (B) Loss and degradation of one entire, replicated parent-daughter DNA double strand (Fig. 6). (C) Degradation of both daughter DNA strands, starting at the replication point and proceeding back toward the origin for some distance (Fig. 6). (D) Same as C, but with degradation starting at a point behind the replication point, leaving a "protected" region of daughter strands in the vicinity of the replication point (Fig. 6). (E) Same as C, but with only one daughter strand degraded (Fig. 6).

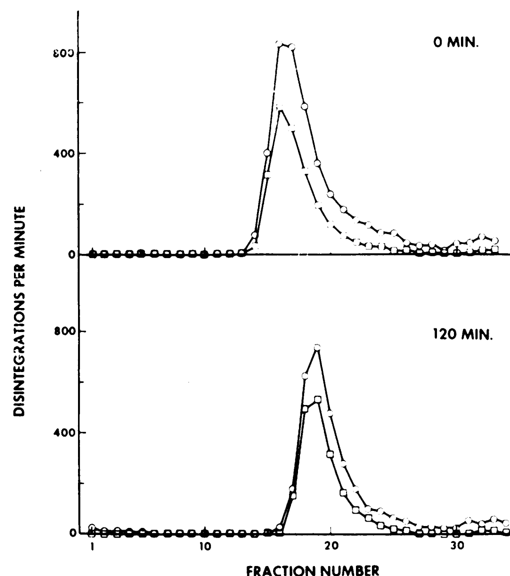

FIG. 4. *Banding of pulse-labeled DNA in CsCl before and after exposure of cells to ACT. A culture of strain 23 thy his was labeled with thymine-2-¹⁴C for 150 min, grown in unlabeled medium for 100 min, labeled with thymine-methyl-³H for 4 min, washed free of label, and supplemented with ACT. At 0 and 120 min, samples were removed and treated* (□, *¹⁴C;* ○, *³H*).

TABLE 2. *Degradation of pulse-labeled DNA in strains 23 thy and 168 thy ind*[a]

Strain	Duration of pulse label (fraction of doubling time)	Fraction of counts degraded (not corrected for nonspecific degradation)
23 *thy*	0.083	0.56
168 *thy ind*	0.077	0.09
168 *thy ind*	0.067	0.11
168 *thy ind*	0.10	0.16

[a] These experiments were identical to those described in Fig. 3, except for the strain of bacteria. In the above order, the average activity of each control culture was 125, 144, 106, and 70 counts per min per sample.

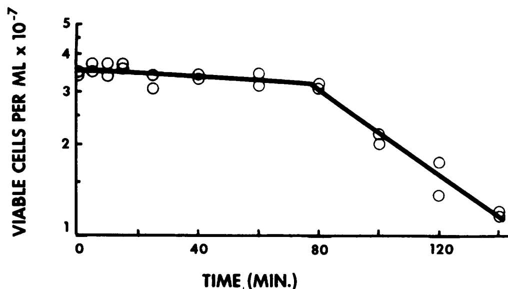

FIG. 5. *Viability of strain 23 thy his in the presence of ACT (10 μg/ml).*

Figure 7 shows the expected curves (*see* Appendix for derivation) for fraction of label degraded plotted against length of labeled region for models B through E as well as the experimentally determined points. Models B and C can be eliminated immediately. Model D does not fit the points well, but can not be definitely eliminated by this method.

Model D was tested by labeling a short segment of DNA and then growing the cells in unlabeled medium for a period sufficient to allow all of the labeled segment to pass out of the hypothetical protected region into the degradable region. If model D were correct, subsequent addition of ACT would be expected to lead to nearly complete degradation of the labeled segment. As seen in Table 3, this was not the case. Thus, model D was eliminated, leaving model E as the working hypothesis.

It is possible to calculate the size of the degraded region assuming that model E is correct. This calculation (Table 4) is based upon the following assumptions: DNA is synthesized at a constant rate throughout the cell cycle, and nonspecific degradation accounts for loss of 11% of the counts from a labeled region of any length at any location. The large value of the result, loss of a single daughter strand equal to 12% of a chromosome in length, argues against the possibility that the replication point alone could be the sensitive region. It is probable, however, that the replication point is the site of initiation of attack.

Fate of parent strand adjacent to decaying daughter strand. Escherichia coli cells which have been deprived of a required amino acid continue to synthesize DNA until previously initiated rounds of DNA synthesis have been completed, at which time DNA synthesis stops (18). When the amino acid is returned to the cells, the chromosomes begin to replicate and remain synchronous for a short time. In histidine-starved cells of strain 23 *thy his*, the amount of DNA initially present increased by about 55% before synthesis stopped (Farmer, *unpublished data*). Restoration of histidine led to immediate resumption of DNA synthesis (Table 5).

Utilizing the above information, an experiment was performed to determine the fate of parental-strand regions which were immediately adjacent to decaying daughter strands. Cells were starved for histidine in order to allow all of the chromosomes to finish previously initiated rounds of DNA replication. The culture was then supplemented with thymine-methyl-^{3}H and histidine and allowed to grow for 10 min before the labeled thymine was removed. After an additional 20 min, the histidine was also removed, and the cells were again starved for histidine for 3 hr. Histidine and thymine-2-^{14}C were added to the suspension and left for 10 min. At this point, the cells were washed and suspended in SGGHT with ACT, and degradation of the labeled DNA was measured as usual. After correction for self-absorption, the tritium counts in the ACT-treated culture had decreased by 11% in 120 min compared with the control, and the ^{14}C counts had decreased by 32%. (The latter corresponds to loss of a single strand equal to 0.096, as calculated in Table 4.) Although the daughter strand decayed normally, the adjacent parent strand did not decay beyond the 11% expected for nonspecific degradation.

Is ACT unique in inducing degradation? The question arose whether the addition of ACT to the cells led to DNA degradation because it was directly involved in the degradative process or because it inhibited DNA synthesis or growth (or both). The results of the five following experiments shed some light on this question.

Effect of thymine starvation. It has been well established that thymine starvation inhibits DNA

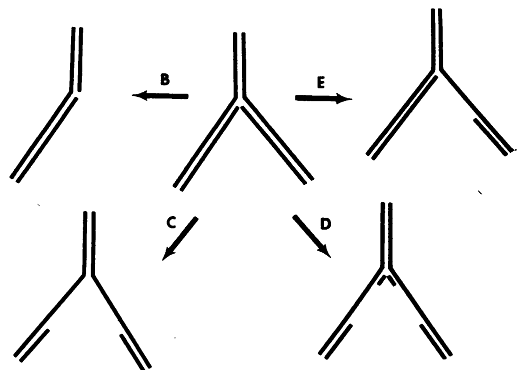

FIG. 6. *Schematic representation of models of degradation of DNA which has been exposed to ACT. Detailed explanation of models is included in the text.*

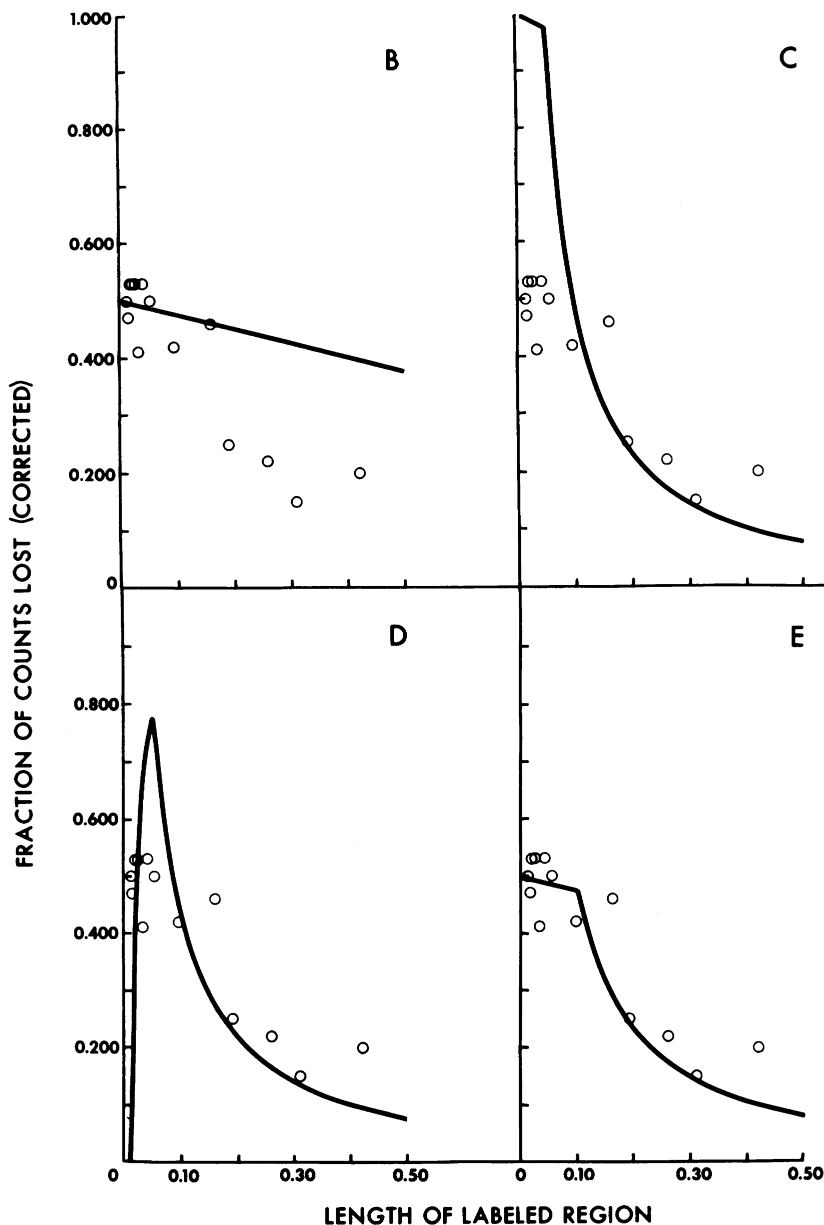

FIG. 7. *Comparison of theoretical curves for models B through E with experimental points. The fraction of counts lost from pulse-labeled DNA after addition of ACT (ordinate) is plotted against the length of the pulse-labeled region (abscissa). The latter is expressed as fraction of a doubling time. Experimental points are from Fig. 3 and Table 2. (Points are corrected for nonspecific degradation by subtracting 0.11 from the experimentally determined value.)*

synthesis very strongly (4). The DNA of thymine-starved cells of strain 23 *thy his* was not degraded (Table 6). In addition, thymine starvation of this strain in the presence of ACT did not alter either the rate or the extent of degradation which is normally seen. The former result agrees with the data of others who showed that thymine starvation of *E. coli* 15 T^- did not lead to breakdown of DNA (26).

Effect of puromycin dihydrochloride. Puromycin is a potent inhibitor of protein synthesis. At the concentration used (30 μg/ml), the increase in cell

TABLE 3. *Effect of a short chase on loss of counts from a short labeled region of DNA*[a]

Duration of pulse label (fraction of doubling time)	Duration of chase (fraction of doubling time)	Fraction of counts degraded (corrected for nonspecific degradation)
0.022	0	0.56
0.022	0	0.38
0.033	0	0.41
0.024	0	0.55
0.014	0	0.37
		0.45 ± 0.094[b]
0.025	0.011	0.43
0.020	0.010	0.34
0.019	0.009	0.61
		0.46 ± 0.14[b]

[a] Cultures were labeled with thymine-*2-¹⁴C* and treated with ACT; the second set was "chased" before ACT treatment by adding unlabeled thymine to a concentration of 100 μg/ml for the interval indicated. These experiments were similar to those described in Fig. 3. In the above order, the average activity of each control culture was 10, 20, 31, 83, 20, 39, 7, and 10 counts per min per sample.

[b] Average plus or minus standard deviation.

TABLE 4. *Calculation of length of degraded region*

Length of labeled region (fraction of doubling time) (L)	Fraction of counts lost (F)	Fraction of counts lost corrected for nonspecific degradation ($F_C = F - 0.11$)	Length of degraded region (fraction of doubling time) ($D = 2 \times F_C \times L$)
0.42	0.31	0.20	0.168
0.31	0.26	0.15	0.092
0.26	0.33	0.22	0.115
0.19	0.36	0.25	0.096
0.16	0.57	0.46	0.147
0.097	0.53	0.42	0.082
0.053	0.61	0.50	—[a]
0.042	0.64	0.53	—
0.033	0.52	0.41	—
0.024	0.67	0.56	—
0.022	0.67	0.56	—
0.014	0.58	0.47	—
0.012	0.61	0.50	—
			0.12 ± 0.035[b]

[a] These samples were labeled for a period less than the calculated length of the degraded region.

[b] Average plus or minus standard deviation.

mass (measured by optical density at 540 mμ) was inhibited by 67% in the first two doubling times after addition of the drug (2 × 47 min). Inhibition of growth by puromycin did not lead to degradation, nor did it prevent degradation in ACT-treated cells (Table 6).

Effect of 9-aminoacridine (9AA). Acridines such as 9AA bind to DNA by intercalation and interfere with DNA and RNA synthesis (13). A very high concentration of 9AA is required to inhibit DNA synthesis in 23 *thy his* (Table 7); even at the highest concentration used, there was only slight degradation of DNA (Table 6).

Effect of phleomycin. Phleomycin has been reported to be a specific inhibitor of the DNA

TABLE 5. *Reinitiation of DNA synthesis after histidine starvation*[a]

Lag period before synthesis resumed	Initial rate of thymine incorporation
min	
0.0	5.0×10^{-12}
0.5	4.2×10^{-12}

[a] Exponentially growing cultures were washed free of histidine, incubated in its absence for 3 hr, and then resupplemented with histidine concurrently with the addition of thymine-*2-¹⁴C*. Each culture was sampled at intervals for acid precipitable counts. The results are expressed as millimicromoles of thymine incorporated per cell-doubling.

TABLE 6. *Effect of various growth-inhibitory treatments on degradation of pulse-labeled DNA*[a]

Treatment of pulse labeled cells after labeling	Length of pulse label (fraction of doubling time)	Fraction of counts lost (uncorrected)	
		−ACT	+ACT
Starved for thymine..	0.065	0.0	0.53
Puromycin dihydrochloride			
(30 μg/ml).........	0.10	0.0	0.29
9-Aminoacridine			
(10 μg/ml).........	0.096	0.0	
(100 μg/ml)........	0.096	0.18	
(1000 μg/ml).......	0.096	0.13	
Phleomycin			
(5 μg/ml)..........	0.073	0.94	
(5 μg/ml)..........	0.083	0.93	
(5 μg/ml)..........	0.26	0.94	
(5 μg/ml)..........	0.26	0.97	
(5 μg/ml) 168 *thy ind*..........	0.086	0.97	

[a] In all experiments except the one indicated, strain 23 *thy his* was used. The conditions of each experiment were those described in Fig. 3, except that the indicated drug or treatment was provided in addition to or in place of ACT. In the above order, the average activity of each control culture was 89, 266, 49, 49, 49, 37, 62, 191, 170, and 49 counts per min per sample.

TABLE 7. *Inhibition of DNA and RNA synthesis in strain 23 thy his by 9-aminoacridine and phleomycin*[a]

Treatment	Concn	Fraction of normal amount of synthesis in 30 min	
		RNA (uracil incorporation)	DNA (thymine incorporation)
	µg/ml		
9-Aminoacridine	10		0.89
	100		0.33
	1,000		0.12
Phleomycin	1	0.64	0.48
	5	0.35	0.04
	20	0.23	0.00

[a] Either thymine-*2-*^{14}*C* or uracil-*2-*^{14}*C* was added to an exponentially growing culture at the same time that the indicated drug was added. The culture was sampled for acid-precipitable counts at intervals. The values given above are the ratios of counts per minute incorporated into treated cultures divided by the counts per minute incorporated into control cultures.

polymerase-catalyzed reaction in vitro (8). This drug interfered with both DNA and RNA synthesis in strain 23 *thy his*, although it inhibited the former more strongly (Table 7). Addition of phleomycin to pulse-labeled cells led to degradation of the labeled region of the DNA, but degradation was complete and did not resemble that seen in the presence of ACT (Table 6). Also in contrast to ACT, phleomycin treatment led to massive degradation in strain 168 *thy ind.* Phleomycin resembles mitomycin in its effect on cellular DNA (15, 26).

Effect of energy deprivation. Since *B. subtilis* is an obligate aerobe, it is possible to deprive cells of energy, and thus inhibit growth, by incubating them under a nitrogen atmosphere. Degradation of pulse-labeled DNA was inhibited almost completely when cells were incubated under nitrogen during exposure to ACT (Fig. 8a). Inhibition of degradation was not due to exclusion of ACT from the cells, as shown by two experiments. (i) Delaying the replacement of air by nitrogen for 10 min after ACT had been added to the cells did not alter the subsequent inhibitory effect of energy deprivation (Fig. 8b); 10 min was sufficient to allow a substantial incorporation of ACT into the cells, as judged by the color of washed cells and by the decay of counts in cells given a very short pulse of labeled thymine (*see*, e.g., Fig. 3). (ii) The pattern of uptake of ACT-methyl-^{14}C was the same (Fig. 9) for cells which were incubated under air or under nitrogen. (The latter were incubated under nitrogen for 5 min before addition of ACT as well as during exposure to it.) Incorporation of ACT was so rapid that the initial kinetics could not be measured. After 10 min, the number of ACT molecules incorporated per cell was approximately 6×10^7 (about 6 ACT molecules per DNA guanine residue).

Clearly, neither inhibition of cell growth nor interruption of DNA synthesis is sufficient to induce DNA degradation of the sort seen in the presence of ACT. The drug must play some active part in the degradative process. What that role might be will be discussed below.

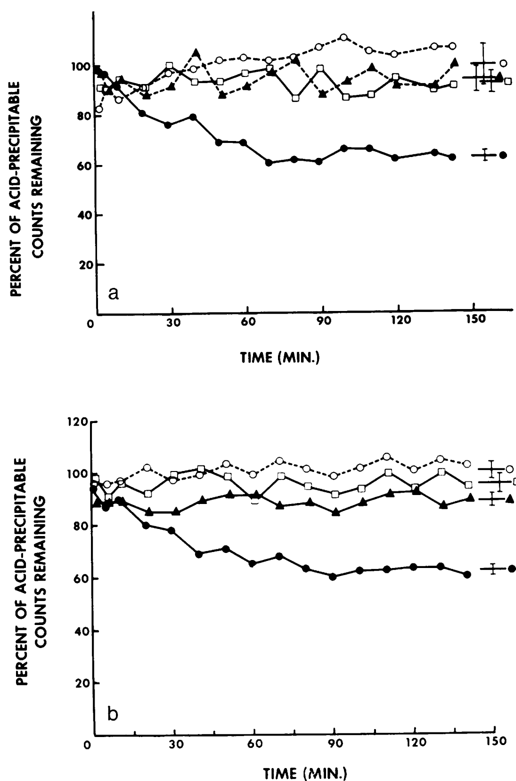

FIG. 8. *Effect of energy deprivation on ACT-induced degradation of DNA. (a) A culture of strain 23 thy his was labeled for 0.067 doubling time and treated with ACT; part of the control culture and part of the ACT-treated culture were bubbled with nitrogen instead of air* (○, *control;* ●, *ACT;* ▲, *control + nitrogen;* □, *ACT + nitrogen; 100% = 58 counts/min*). (*b*) *Cells were labeled for 0.11 doubling time and treated with ACT as above, except that a part of the ACT-treated culture was incubated with air for 10 min and then bubbled with nitrogen for the remainder of the experiment* (○, *control;* ●, *ACT;* □, *ACT + nitrogen;* △, *ACT + nitrogen after 10 min; 100% = 57 counts/min*). *Horizontal and vertical bars show mean ± 1 standard deviation, respectively. Mean of ACT does not include points to the left of 60 min.*

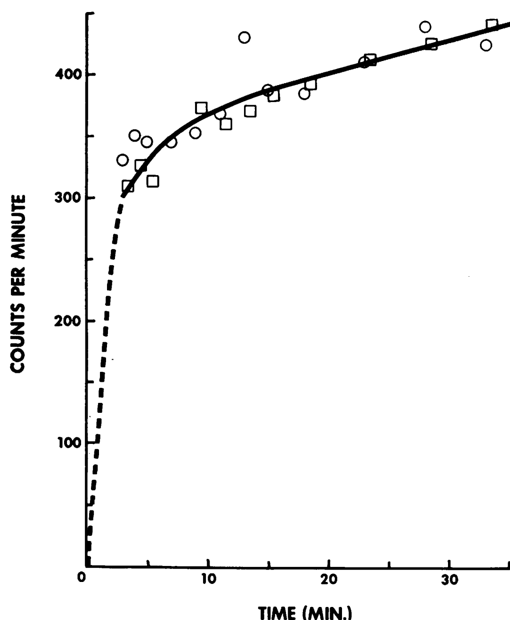

FIG. 9. *Uptake of ACT in the presence and absence of air. A culture of 23 thy his was divided, one half being incubated under nitrogen and the other under air. After 5 min, ACT-methyl-^{14}C was added to each culture (with sufficient unlabeled ACT to bring the concentration to 10 μg/ml). At intervals, samples were removed, filtered (Millipore HAWG023), washed with 5 ml of cold medium, and assayed for radioactive counts (□, air; ○, nitrogen).*

DISCUSSION

The effect of large doses of ACT on bacterial cells does not resemble that of mitomycin C, which leads to massive destruction of cellular DNA and very rapid loss of viability (15, 26). The effect of nalidixic acid (5) is somewhat similar to that reported here for ACT, but the published data are not sufficient to make a detailed comparison.

A working hypothesis, which is currently being tested, has been adopted to explain the mechanism of degradation. It is as follows. ACT binds to the DNA in some unspecified manner, such that the replication point is physically altered, freeing the ends of the daughter strands for attack by an exonuclease. Degradation of only one daughter strand could be due to specificity of the exonuclease for only one of the two chemically distinct, free ends. The limitation of degradation to 12% of a chromosome length could be due to random termination of the degradative process, the observed value being the mean value for the population. The requirement for energy remains unexplained, although it is possible to construct a number of very hypothetical models.

The absence of degradation in strain 168 *thy ind* can best be explained as due to the absence of the hypothetical exonuclease from cells of this strain, either because the structural gene is absent, or because it is inactive. (The exonuclease must be synthesized constitutively in 23 *thy his*, since it could not be induced after addition of ACT.)

There is one precedent for an energy-stimulated exonuclease. Exonuclease II of *E. coli*, which is inseparable from DNA polymerase, is stimulated when all four of the deoxynucleotide triphosphates are present (19). The nature of the stimulatory effect is not known, although it appears not to be due to allosteric effects. DNA polymerase of *B. subtilis* strain SB 19 has been reported to be free of nuclease activity, although it was not assayed in the presence of the deoxynucleotide triphosphates (24). It has been speculated that exonuclease II may be involved in some way in the replication of DNA (19). If in fact the ACT-induced degradation in strain 23 *thy his* is due to an exonuclease which is analogous to exonuclease II, one would be forced to assume that such exonuclease activity is not required for DNA synthesis, since it is not present in strain 168 *thy ind.* Breakdown of DNA in *B. subtilis* during dark repair of UV damage is an energy-dependent process (27), but the relation of this observation to the degradation reported in this paper is not obvious.

APPENDIX

The calculation of the fraction of counts lost from a pulse-labeled region for each of the models is straightforward except for two corrections. One correction, P, is the fraction of the label which is contained in parental DNA strands owing to chromosomes which complete replication during the labeling period. This fraction of label is thus removed from the pool of label which is degradable. The second correction, Q, is the fraction of the label which is contained in daughter strands whose length is less than the average length of the degraded region, owing to chromosomes which began a new round of replication late in the labeling period. All of the label in this fraction will be degraded.

Derivation of P. For a large, randomly replicating population of DNA molecules, the fraction of label, f_T, which is contained between the terminus of the average molecule and a point a distance s proximal to the terminus, is given by the expression

$$f_T = s/L$$

where L is the total length of a completed DNA molecule. For the calculation of P, we are interested in a region of length, $s = r$, where r is the length of the labeled region (taken as equal to the percentage of a doubling time that label is present in the medium, L thus being equal to 100). Since half of the label in the

fraction f_T will be contained in DNA molecules which did not replicate past the terminus during the labeling period, P will be equal to $f_T/2$, or

$$P = r/2L$$

which is valid for all values of r less than L.

Derivation of Q. The amount of label, $u(a)$, which is contained within daughter strands which began replicating at the origin of replication sometime during the labeling period and which were replicated for a length less than or equal to an arbitrary length a, where a is less than r, is given by the expression

$$u(a) = (a) + (a - 1) + \cdots + (2) + (1) = \sum_{n=0}^{a-1} (a - n)$$

The total amount of label which is contained in the region from the origin to a is equal to $b \cdot r$, where b is a dimensionless number which is numerically equal to a. Therefore, the fraction of label, f_u, within the region between the origin and a which has not replicated beyond a is given by the expression

$$f_u = \frac{u(a)}{b \cdot r}$$

The fraction of the total label which is contained in the length a is equal to a/L. If a is taken to be equal to the length of the degraded region, then Q is given by the expression

$$Q = \frac{\sum_{n=0}^{a-1} (a - n)}{b \cdot r} \cdot \frac{a}{L}$$

The quantity a was varied over a wide range in the calculations. The theoretical curves shown in Fig. 7 are for the values of a which gave the best fit for each model to the experimental points.

The fraction of counts lost, F, can then be calculated as follows:

Model B $\quad F_B = 0.5(1 - P)$

Model C

$$F_C = (a/r)(1 - P - Q) + Q \qquad a \leq r$$

$$F_C = 1 - P \qquad a \geq r$$

Model D

$$F_D = (F_C)(r - 1)/r \qquad r \geq 1$$

$$F_D = 0 \qquad r \leq 1$$

(the protected region is set equal to 1% of a chromosome in length on the basis of the data in Fig. 3)

Model E $\quad F_E = 0.5\, F_C$

ACKNOWLEDGMENTS

I am greatly indebted to Frank Rothman for many helpful discussions and for critical review of the manuscript. I thank Kenneth Anselmo for technical assistance.

This investigation was supported by a grant from the National Foundation and by National Science Foundation grant GB 5892. This work was begun in the course of a predoctoral fellowship supported by Public Health Service training grant HD-00019.

LITERATURE CITED

1. ANAGNOSTOPOULOS, C., AND J. SPIZIZEN. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. CAVALIERI, L. F., AND R. G. NEMCHIN. 1964. The mode of interaction of actinomycin D with deoxyribonucleic acid. Biochim. Biophys. Acta **87**:641–652.
3. CERAMI, A., E. REICH, D. C. WARD, AND I. H. GOLDBERG. 1967. The interaction of actinomycin with DNA: requirement for the 2-amino group of purines. Proc. Natl. Acad. Sci. U.S. **57**:1036–1042.
4. COHEN, S. S., AND H. D. BARNER. 1954. Studies on unbalanced growth in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **40**:885–893.
5. COOK, T. M., K. G. BROWN, J. V. BOYLE, AND W. A. GOSS. 1966. Bactericidal action of nalidixic acid on *Bacillus subtilis*. J. Bacteriol. **92**:1510–1514.
6. ELLIOTT, W. H. 1962. The effects of antimicrobial agents on deoxyribonucleic acid polymerase. Biochem. J. **86**:562–567.
7. ERON, L. J., AND B. R. MCAUSLAN. 1966. Inhibition of deoxyribonuclease action by actinomycin D and ethidium bromide. Biochim. Biophys. Acta **114**:633–636.
8. FALASCHI, A., AND A. KORNBERG. 1964. Phleomycin, an inhibitor of DNA polymerase. Federation Proc. **23**:940–945.
9. FARMER, J. L., AND F. ROTHMAN. 1965. Transformable thymine-requiring mutant of *Bacillus subtilis*. J. Bacteriol. **89**:262–263.
10. GELLERT, M., C. E. SMITH, D. NEVILLE, AND G. FELSENFELD. 1965. Actinomycin binding to DNA: mechanism and specificity. J. Mol. Biol. **11**:445–457.
11. GOLDBERG, I. H., M. RABINOWITZ, AND E. REICH. 1962. Basis of actinomycin action. I. DNA binding and inhibition of RNA-polymerase synthetic reactions by actinomycin. Proc. Natl. Acad. Sci. U.S. **48**:2094–2101.
12. HAMILTON, L. D., W. FULLER, AND E. REICH. 1963. X-ray diffraction and molecular model building studies of the interaction of actinomycin with nucleic acids. Nature **198**:538–540.
13. HURWITZ, J., J. J. FURTH, M. MALAMY, AND M. ALEXANDER. 1962. The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of the enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by actinomycin and proflavin. Proc. Natl. Acad. Sci. U.S. **48**:1222–1230.
14. KEIR, H. M., H. OMURA, AND J. B. SHEPHERD. 1963. Inhibition of a mammalian deoxyribo-

nucleic acid nucleotidyltransferase by actinomycin D. Biochem. J. **89**:425–430.
15. KERSTEN, H., AND H. M. RAUEN. 1961. Degradation of deoxyribonucleic acid in *Escherichia coli* cells treated with mitomycin C. Nature **190**: 1195–1196.
16. KERSTEN, W., H. KERSTEN, AND W. SZYBALSKI. 1966. Physicochemical properties of complexes between deoxyribonucleic acid and antibiotics which affect ribonucleic acid synthesis (actinomycin, daunomycin, cinerubin, nogalamycin, chromomycin, mithramycin, and olivomycin). Biochemistry **5**:236–244.
17. LABOURET, T., J. POUYET, AND P. FROMAGEOT. 1965. Note sur l'interaction entre actinomycine C1 et DNA. Bull. Soc. Chim. Biol. **47**:1579–1590.
18. LARK, K. G. 1966. Regulation of chromosome replication and segregation in bacteria. Bacteriol. Rev. **30**:3–32.
19. LEHMAN, I. R. 1967. Deoxyribonucleases: their relationship to deoxyribonucleic acid synthesis. Ann. Rev. Biochem. **36**:645–688.
20. LEITH, J. D., JR. 1963. Acridine orange and acriflavin inhibit deoxyribonuclease action. Biochim. Biophys. Acta **72**:643–644.
21. LIERSCH, M., AND G. HARTMANN. 1964. Die Bindung von Proflavin und Actinomycin an Desoxyribonucleinsäure. I. Der Einfluss der Ionenstärke und des pH sowie die Wirkung von Harnstoff auf die Bindung. Biochem. Z. **340**:390–399.
22. LIERSCH, M., AND G. HARTMANN. 1965. Die Bindung von Proflavin und Actinomycin an Desoxyribonucleinsäure. II. Die Bindung an denaturierte und einsträngige DNA, Apyrimidinsäure und Apurinsäure. Biochem. Z. **343**: 16–28.
23. MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
24. OKAZAKI, T., AND A. KORNBERG. 1964. Enzymatic synthesis of deoxyribonucleic acid. XV. Purification and properties of a polymerase from *Bacillus subtilis*. J. Biol. Chem. **239**:259–268.
25. REICH, E. 1964. Actinomycin: correlation of structure and function of its complexes with purines and DNA. Science **143**:684–689.
26. REICH, E., A. J. SHATKIN, AND E. L. TATUM. Bactericidal action of mitomycin C. Biochim. Biophys. Acta **53**:132–149.
27. REITER, H., B. STRAUSS, R. MARONE, AND M. ROBBINS. 1966. Actinomycin D inhibition of UV-dark repair in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **24**:892–898.
28. ROLFE, R. 1967. On the mechanism of thymineless death in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S. **57**:114–121.
29. SARKAR, N. K. 1967. Effects of actinomycin D and mitomycin C on the degradation of deoxyribonucleic acid and polydeoxyribonucleotide by deoxyribonucleases and venom phosphodiesterase. Biochim. Biophys. Acta **145**:174–177.
30. SOSKA, J., AND K. G. LARK. 1966. Regulation of nucleic acid synthesis in *Lactobacillus acidophillus* R-26. Biochim. Biophys. Acta **119**:526–539.