[11] Huehns, E. R., and E. M. Shooter, *J. Mol. Biol.*, **3**, 257 (1961).
[12] Huisman, T. H. J., and C. A. Meyering, *Clin. Chim. Acta*, **5**, 103 (1960).
[13] Smithies, O., *Biochem. J.*, **71**, 585 (1959).
[14] Riggs, A., unpublished observation.
[15] Muller, C. J., *Nature*, **186**, 643 (1960).
[16] Rossi-Fanelli, A., and E. Antonini, *Arch. Biochem. Biophys.*, **77**, 478 (1958).
[17] Wyman, J., *Adv. Prot. Chem.*, **4**, 407 (1948).
[18] Benesch, R., and R. E. Benesch, *J. Biol. Chem.*, **236**, 405 (1961).
[19] Jones, R. T., W. A. Schroeder, J. E. Balog, and J. R. Vinograd, *J. Am. Chem. Soc.*, **81**, 3161 (1959).
[20] Benesch, R. E., H. M. Ranney, R. Benesch, and G. M. Smith, *J. Biol. Chem.*, **236**, 2926 (1961).
[21] Muller, C. J., *Molecular Evolution* (Assen, Netherlands: Van Gorcum, 1961).

*DIFFERENTIAL DESTRUCTION OF THE TRANSFORMING ACTIVITY OF DAMAGED DEOXYRIBONUCLEIC ACID BY A BACTERIAL ENZYME**

BY BERNARD S. STRAUSS

DEPARTMENT OF MICROBIOLOGY, THE UNIVERSITY OF CHICAGO

Communicated by W. H. Taliaferro, July 10, 1962

When cells of *Bacillus subtilis* were treated with the mutagenic alkylating agent methyl methanesulfonate and then incubated in a growth medium, the total deoxyribonucleic acid (DNA) either failed to increase or decreased slightly. During the same period, C^{14}-thymidine was continuously incorporated into an alkali-resistant, hot acid-soluble form identified thereby as DNA.[1] These observations suggested that the DNA in treated cells was subject to some sort of degradative process since nontreated cells increased in total DNA from the start of incubation. It now appears that both alkylation and ultraviolet irradiation alter the DNA of *B. subtilis* in a way which makes it differentially susceptible to inactivation by an enzyme present in extracts of *B. subtilis* and *Micrococcus lysodeikticus*.

Materials and Methods.—Transforming DNA was prepared from *B. subtilis* by the method of Marmur.[2] The DNA was alkylated at a concentration of 0.25 mg per ml in 0.1 *M* phosphate buffer, pH 7.0, by the addition of redistilled methyl methanesulfonate to give a final concentration of 0.025 *M* for the alkylating agent. After reaction at 37°C, the DNA was precipitated by the addition of 2 volumes of cold 95% ethanol; the precipitated DNA was lifted out on a glass rod, drained, dissolved in 0.15 *M* sodium chloride-0.015 *M* sodium citrate (saline-citrate)[2] and reprecipitated. The DNA was finally stored in saline-citrate in the cold.

M. lysodeikticus cells were grown on the medium described by Steiner and Beers[3] and stored at −25°C. Extracts were prepared by lysis of a 2% cell suspension with lysozyme (0.5 mg/ml) followed by centrifugation in the cold at 18,000 *g*. The supernatant was then used. Alternately, and as indicated below, a 10% cell suspension was treated with lysozyme, after which the lysate was subjected before centrifugation to sonic disintegration for 5 min in a Raytheon 10 kc sonic oscillator. Extracts of *B. subtilis* were prepared in a similar manner.

Enzyme reactions were carried out in a one ml total volume containing transform-

ing DNA, 10 μM $MgCl_2$ and 0.02 *M* phosphate buffer, pH 7.5. The reaction was stopped by the addition in sequence of 0.25 ml of 25% sodium lauryl sulfate, 0.5 ml of 5 *M* sodium perchlorate, 0.75 ml of saline-citrate, 6 ml of chloroform, and 0.3 ml of isoamyl alcohol, after which the mixture was shaken for 30 min in the cold. The resulting emulsion was separated by centrifugation and the clear supernatant was taken off and stored in the cold until assayed. This procedure was adopted because of the heat sensitivity of alkylated DNA.[4] Transformation assays were performed with an indole-requiring strain by the method of Anagnostopoulos and Spizizen.[5]

Results.—Alkylated transforming DNA prepared from wild-type *B. subtilis* was differentially inactivated when incubated with an extract from *M. lysodeikticus* (Table 1). There was no such differential sensitivity when control and alkylated

TABLE 1

SUSCEPTIBILITY OF TRANSFORMING ACTIVITY IN NORMAL AND ALKYLATED *B. subtilis* DNA TO THE ACTION OF *M. lysodeikticus* EXTRACT

DNA preparation	Incubation with *M. lysodeikticus* extract (time at 37°, min.)	Extract	Specific transforming activity
Control	0	Fresh	137*
	30	"	175
	60	"	140
	60	Boiled	130
Alkylated for 60 min with 0.025 *M* methyl methanesulfonate	0	Fresh	25.4
	30	"	2.7
	60	"	2.1
	60	Boiled	7.9

* Recorded transformants per unit optical density (259 mμ) $\times 10^{-4}$. 2.1×10^7 cells were plated for the determination of transformants after treatment with extracts from a 2% suspension of *M. lysodeikticus* cells. The DNA was diluted in all experiments to give an optical density at 259 mμ of about 0.2 in the reaction mixture.

DNA were incubated with pancreatic deoxyribonuclease or with a cobra venom preparation. Incubation for 15 min with 0.2 mg of lyophilized cobra venom (*Naja ophiophagus*) reduced the activity of control DNA 73-fold and of alkylated DNA 125-fold. A boiled venom preparation resulted in a 1.8-fold decrease in transforming activity of the alkylated sample.

Differential sensitivity of alkylated DNA was obtained with *B. subtilis* preparations, but since these extracts inactivated unaltered transforming DNA (Table 2), most of the experiments employed preparations from *M. lysodeikticus*. Fresh extracts of this organism do not inactivate unreacted *B. subtilis* transforming DNA, and an ammonium sulfate precipitable fraction from *M. lysodeikticus* which inactivated alkylated transforming DNA led to an increase of 11 per cent in acid-soluble phosphorus when it was incubated for 30 min at 37°C in the absence of added nucleotides with a heavily P^{32}-labeled DNA preparation from *B. subtilis*.

TABLE 2

INACTIVATION OF TRANSFORMING DNA BY INCUBATION WITH BACTERIAL EXTRACTS

	Transformants per unit of optical density (259 mμ) $\times 10^{-4}$ Min at 37°C	
	0	60
Extract of a 2% cell suspension of:		
Micrococcus lysodeikticus	24.1	21.8
Bacillus subtilis, indole-requiring	30.0	6.8

3.7×10^7 cells were plated for the transformation assay.

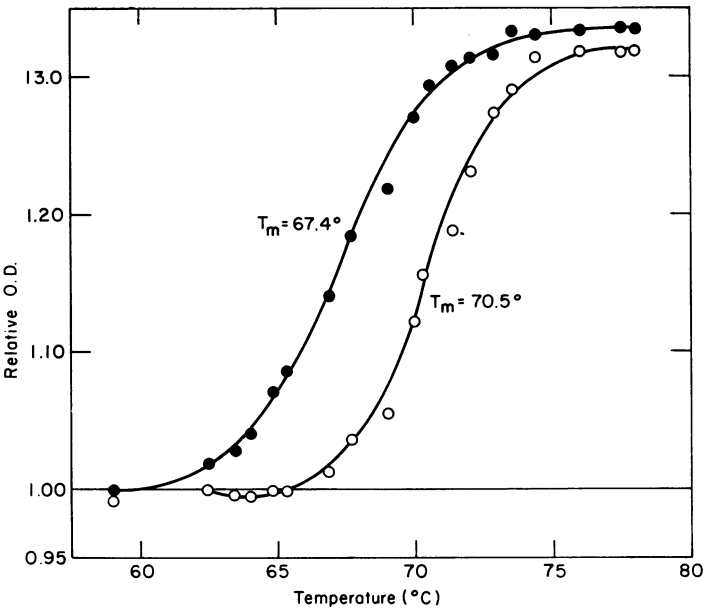

FIG. 1.—Thermal denaturation curves of control and alkylated DNA. *Bacillus subtilis* DNA was treated at 37° with 0.025 *M* methyl methanesulfonate as described in the text. A sample of the DNA dissolved in saline-citrate was diluted 1:20 in a pH 7.25 buffer (5×10^{-3} *M* NaCl, 10^{-3} *M* phosphate, and 10^{-4} *M* sodium ethylenediaminetetracetate). Optical density was measured at 259 mμ at the indicated temperature in a Beckman DU spectrophotometer. Solid circles represent DNA alkylated for 90 minutes. Open circles represent non-treated DNA.

DNA alkylated with 0.025 *M* methyl methanesulfonate for 60 or 90 min at 37°C gave as great a hyperchromic effect as did untreated DNA (Fig. 1). This alkylated DNA lost its biological activity on heating for 5 hr at 50°C.[4] The loss of activity occurred with less than 2% increase in absorption at 259 mμ. A 25 per cent increase in optical density occurred when an alkylated DNA preparation heated for 5 hr at 50°C in saline-citrate was heated at 100°C for 10 min and cooled by plunging into an ice bath. These data show that the alkylated DNA used in these experiments was not denatured by either the alkylation or the subsequent heating at a temperature below the "melting point." There was a lowering of the "melting point" of alkylated DNA (Fig. 1) similar to that observed after ultraviolet irradiation of DNA.[6]

The inactivation of alkylated transforming DNA by heat was additive to the inactivation by the enzyme just described. An alkylated DNA sample maximally inactivated by enzyme treatment remained heat-sensitive (Table 3), and the transforming activity remaining after a 5-hr heating period at 50°C was sensitive to inactivation by a sonic extract of *M. lysodeikticus*. The amount of degradation depended on the amount of alkylation (Table 4). Samples of transforming DNA treated for 90 min with methyl methanesulfonate lost a higher proportion of their activity than did samples alkylated for shorter periods.

Irradiation with ultraviolet light sensitized transforming DNA to inactivation by extracts of *M. lysodeikticus*, and this sensitization appeared to be dose-dependent (Fig. 2). In contrast to alkylated DNA, the ultraviolet-irradiated DNA was not

TABLE 3

EFFECT OF AN EXTRACT OF *M. lysodeikticus* AND OF HEATING ON THE TRANSFORMING ACTIVITY OF METHYLATED *B. subtilis* DNA

Minutes incubation at 37° with 0.5 ml extract from a 2% cell suspension	Control, not alkylated		Alkylated	
	Not heated	Incubated with enzyme and then heated 5 hr at 50°C	Not heated	Incubated with enzyme and then heated 5 hr at 50°C
0	109*	110	27.5	2.1
15	86.2	117	1.0	0.059
Not incubated/incubated	1.3	0.94	27.5	36.

* Transformants per unit optical density (259 mμ) × 10^{-4}.
DNA was alkylated for 60 min at 37°C with 0.025 *M* methyl methanesulfonate. Specific transforming activities were calculated from the number of transformants obtained at dilutions of DNA at which the transformation response to DNA concentration is known to be linear. Incubation of alkylated samples with extract for additional periods up to 60 min resulted in no further decrease in activity. 9 × 10^6 cells were plated for the transformation assay.

TABLE 4

EFFECT OF THE EXTENT OF ALKYLATION ON THE SENSITIVITY OF TRANSFORMING *B. subtilis* DNA TO INACTIVATION BY AN EXTRACT OF *M. lysodeikticus*

Minutes incubation at 37° with 0.5 ml crude extract from a 2% cell suspension	Time alkylated (min)			
	0	30	60	90
0	79.5*	34.3	25.8	19.6
15	45.0	20.1	2.9	0.61
Not incubated/incubated	1.8	1.7	8.9	32

* Transformants per unit optical density (259 mμ) × 10^{-4}.
DNA was alkylated as described in the text. 1.2 × 10^7 cells were plated for the transformation assay.

sensitive to heat treatment for 5 hr at 50°C. The residual transforming activity of heat-denatured DNA was somewhat susceptible to enzymatic inactivation. Denatured DNA with 10 per cent of its original transforming activity was prepared by immersion of a solution of *B. subitilis* DNA in 0.1 *M* phosphate buffer, pH 7.0, in a boiling water bath for 10 min, after which the solution was cooled by immersion in an ice bath. This preparation was reduced in transforming activity 4.1-fold by 15 min incubation at 37°C with an active ammonium sulfate enzyme fraction compared to a 1.9-fold reduction in activity of the control DNA and a 14-fold reduction in the activity of DNA irradiated with ultraviolet light to give 68 per cent of its original transforming activity.

Some preliminary attempts were made to study the characteristics of the substance responsible for the inactivation of alkylated DNA. The material was precipitated by protamine sulfate and could be eluted from the protamine sulfate by 0.07 *M* phosphate buffer, pH 8.0, and precipitated by ammonium sulfate as described by Lehman[7] for the preparation of the phosphodiesterase from *Escherichia coli*. Since the addition of protein denaturing agents prevented degradation of alkylated DNA and since the activity was susceptible to salting out, it was assumed that the activity was due to enzyme action. However, the activity was not completely destroyed by boiling. In one experiment using the fraction obtained after ammonium sulfate precipitation, there was a 57-fold reduction in activity on incubation of alkylated DNA with enzyme and an 11-fold reduction when the alkylated DNA was incubated with a boiled portion of the same enzyme extract (cf. also Table 1). The activity of the ammonium sulfate precipitate dissolved in 0.02 *M* phosphate buffer, pH 7.5, could be preserved at −25°C.

Discussion.—These experiments show that alkylation and ultraviolet irradiation can sensitize DNA to further degradation without destroying the biological activity of the DNA since the sensitive DNA in these experiments is still capable of trans-

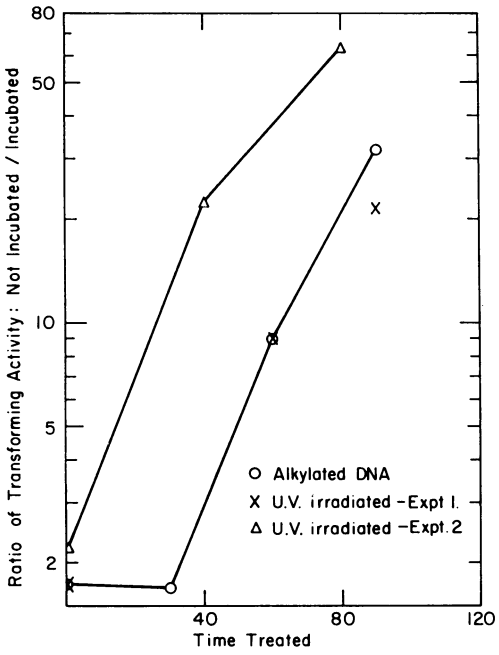

FIG 2.—Ultraviolet dose dependence of the degradative reaction. Methylated DNA: (O) min treated with 0.025 *M* methyl methanesulfonate as described in the text. The treated DNA was incubated 15 min with a crude *M. lysodeikticus* extract. Ultraviolet irradiation (experiment 1): (×) sec irradiation 50 cm from a 15-watt germicidal lamp. After 90 sec, the DNA had a transforming activity 36 per cent of the untreated control. Incubation for 30 min with a crude *M. lysodeikticus* extract. Ultraviolet irradiation (experiment 2): (Δ) sec irradiation 45 cm from a 15-watt germicidal lamp. After 80 sec irradiation, the DNA had a residual transforming activity 25 per cent of the untreated control. Incubation for 30 min with an ammonium sulfate precipitable fraction from *M. lysodeikticus*.

formation. The measurement is therefore of a different kind of damage than that studied with the photoreactivation enzyme.[8] The photoreactivation studies were concerned with the restoration of activity in DNA sufficiently damaged so that it was not efficient in transformation.

It is not possible to specify the structural change induced by alkylation and ultraviolet irradiation which results in sensitization to enzymatic inactivation of that fraction of the DNA which retains biological activity, nor is there any information about the chemical nature of the products of enzymatic action. Ultraviolet-irradiated DNA is sensitive to enzymatic inactivation but is not inactivated by heating at 50°C. The transforming activity remaining after enzymatic inactivation of alkylated DNA retains its susceptibility to heating at 50°C and, conversely, the activity of alkylated DNA remaining after heating for 5 hr at 50°C is susceptible to enzyme action. The inactivation of alkylated DNA by heating below the "melting" temperature is most likely due to depurination reactions.[9] The data can be restated by saying that there may be sites on the alkylated DNA which are susceptible to heat inactivation although resistant to enzymatic inactivation and that ultraviolet irradiation can damage the DNA molecule in a way which makes it susceptible to enzymatic inactivation without introducing the heat susceptibility.

These experiments suggest that the breakdown of DNA seen in many cells after irradiation[10] could be due to activation of the DNA substrate rather than to activation of a deoxyribonuclease. It is also possible that the process of "mutation frequency decline,"[11] which is supposed to be enzymatic, might represent a degradation of partially damaged but not inactivated DNA *in vivo* by an enzyme system similar to that reported here.

I should like to acknowledge the expert technical assistance of Joan Kerst. My thanks are

due to J. W. Moulder, R. Haselkorn, and E. P. Geiduschek for their suggestions and help in preparation of the manuscript.

* Supported in part by grants from the National Science Foundation and from the National Institutes of Health. The support of the Abbott Memorial Fund of the University of Chicago is gratefully acknowledged.

[1] Strauss, B., *J. Gen. Microbiol.*, in press.

[2] Marmur, J., *J. Molec. Biol.*, **3**, 208 (1961).

[3] Steiner, R., and R. Beers, *Polynucleotides* (Amsterdam: Elsevier, 1961), p. 374.

[4] Strauss, B., *Bacteriol. Proc.*, **G38**, 44 (1962).

[5] Anagnostopoulos, C., and J. Spizizen, *J. Bacteriol.*, **81**, 741 (1961).

[6] Marmur, J., W. Anderson, L. Matthews, K. Berns, E. Gajewska, D. Lane, and P. Doty, *J. Cell. Comp. Physiol.*, **58**, suppl. 1, 33 (1961).

[7] Lehman, I. R., *J. Biol. Chem.*, **235**, 1479 (1960).

[8] Rupert, C., *J. Gen. Physiol.*, **43**, 573 (1960).

[9] Bautz, E., and E. Freese, these PROCEEDINGS, **46**, 1585 (1960); Brookes, P., and P. Lawley, *Biochem. J.*, **80**, 496 (1961); Strauss, B., *J. Bacteriol.*, **83**, 241 (1962).

[10] Stuy, J., *Radiation Research*, **14**, 56 (1961).

[11] Doudney, C., and F. Haas, these PROCEEDINGS, **45**, 709 (1959).

---

*LINEAR BIOSYNTHESIS OF TOBACCO MOSAIC VIRUS: EVIDENCE THAT SHORT VIRUS RODS ARE NATURAL PRODUCTS OF TMV BIOSYNTHESIS**

BY JANEY SYMINGTON, BARRY COMMONER, and MAS YAMADA

THE HENRY SHAW SCHOOL OF BOTANY AND THE ADOLPHUS BUSCH III LABORATORY OF MOLECULAR BIOLOGY, WASHINGTON UNIVERSITY, ST. LOUIS

Communicated by Frits W. Went, July 30, 1962

This paper considers whether the ribonucleoprotein rods that are the distinctive product of the tobacco mosaic virus (TMV) infection process are inherently variable in length. The problem is of interest because it influences conclusions regarding the mechanisms of TMV biosynthesis and replication.

In previous studies of the incorporation of C^{14} into TMV in the infected tobacco leaf we have shown that the virus protein and RNA components are synthesized concurrently from the leaf's pools of free amino acids and ribonucleotides.[1] This observation, which has now been confirmed in two other RNA-bearing viruses, poliovirus[2] and fowl plague virus,[3] mitigates against the possible occurrence of free RNA and protein precursors of TMV, and suggests instead that incomplete or alternative forms of the virus are more likely to consist of ribonucleoprotein. Since the virus' protein subunits and RNA fiber form coaxial structures in the TMV rod, any ribonucleoprotein alternative to the predominant 3,000 Å rod is likely to take the form of a rod of some different length.

The problem of TMV rod length has been the subject of considerable study, but there is little agreement as to the significance of the results. While it is now clear that rods longer than 3,000 Å are preparative artefacts,[4, 5] there is wide disagreement regarding the origin of the rods shorter than 3,000 Å that are always observed in TMV preparations. Two recent reviews of this question reach opposite conclusions. Pirie[6] supports the view that short rods are natural products of TMV bio-