*ON THE INACTIVATION OF TRANSFORMING DNA BY TEMPERATURES BELOW THE MELTING POINT**

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In our studies on the heat inactivation at neutral pH of transforming principle prepared by employing water-saturated phenol as a deproteinizing agent, we have consistently observed exponential heat survival curves at subcritical temperatures, i.e., below the melting point (denaturation).[1] On the other hand, using DNA deproteinized by chloroform–amyl alcohol, we and others[4, 5] observe a survival curve which is concave downward, having a finite initial slope which increases with time of heating.

Since there is a growing interest in the study of heat sensitivity of different markers residing in DNA molecules,[1, 3–6] we wish to describe our experiments showing that the present difference in the behavior under heat is due to the method of preparation of the DNA. The data shown here are for heat-induced loss of the capacity to transform pneumococcus to streptomycin resistance. Similar results were found with three other drug-resistance markers.

The source of the transforming DNA employed in this investigation is a pneumococcal strain recently obtained from Dr. J. Marmur of Harvard University. The strain possesses several unlinked markers, including those for resistance to streptomycin, bryamycin, novobiocin, and erythromycin.

DNA from this strain was prepared by lysing collected cells with desoxycholate and shaking with a chloroform-isoamyl alcohol mixture. The aqueous phase was separated by centrifugation, removed and treated with boiled ribonuclease, following which the chloroform step was repeated, the DNA precipitated with ethanol, and the chloroform and precipitation steps repeated again. The DNA was redissolved in 30 ml of a standard saline-citrate solution and then dialyzed exhaustively against various salt concentrations, ending up in standard saline-citrate. Three weeks later, this preparation was treated with trypsin and chymotrypsin and shaken again with chloroform-isoamyl alcohol, and the aqueous phase was again exhaustively dialyzed against saline-citrate plus 0.01 *M* Tris, pH 8.7. This material behaved reproducibly in the ultracentrifuge and had an $S^{50}_{20,w}$ of 25 Svedbergs. This stock is hereafter referred to as "Sevaged TP."

As a control measure, an aliquot of the above stock solution was shaken manually with an equal volume of water-saturated phenol for 10 minutes; the aqueous phase was separated by centrifugation and precipitated with two volumes of ethanol. The alcohol precipitate was centrifuged and the pellet washed once with 65 per cent ethanol in water to minimize contamination with phenol and taken up in sterile 0.1 *M* phosphate buffer, pH 7.25. The final concentration estimated by the method of Keck[7] was adjusted to 50 μg DNA/ml. This stock is hereinafter designated "Phenoled TP."

A third preparation of DNA, on the heat inactivation of which we have already conducted a series of studies,[1] was extracted from a streptomycin-resistant transformant of pneumococcus strain Rx-1, which originally came from the laboratory

of Dr. Arnold Ravin, University of Rochester, and has been cultured for several years at Yale. A cell suspension was lysed with sodium desoxycholate and shaken with an equal volume of water-saturated phenol for 20 minutes at room temperature, and the two liquid phases were separated by centrifugation. The aqueous supernatant was carefully separated from the denatured protein interphase and the lower phenol phase. The cycle of phenol treatment was repeated once and the aqueous phase bearing the bulk of the DNA was alcohol-precipitated with 2 volumes of ethanol. The precipitate was wound on a glass rod and redissolved in neutral 0.1 M phosphate buffer containing 10^{-3} M citrate. The resulting solution was again alcohol-precipitated and the precipitate handled in the same manner as described above. The resulting DNA solution was filtered through sterile ultrafine sintered glass filter. The water-clear filtrate was diluted with 0.1 M phosphate buffer, pH 7.25, to make a stock solution of 60 μg-DNA/ml (hereinafter designated III-DNA-A). This preparation differed then from the Sevaged and Phenoled TP in these major respects: (1) it had not been treated with RNase or with chloroform-isoamyl alcohol; (2) it had been extracted from a pneumococcus strain which had been cultured for many years separately from the strain from which the Sevaged and Phenoled TP were derived; and (3) it had been put through an ultrafine filter.

Heat inactivation experiments were conducted in 0.1 M sodium phosphate buffer, pH 7.25 (measured at room temperature with a Beckman model H pH meter), in a water bath the temperature of which was controlled to within 0.1°. The concentration of DNA during heating was varied according to the experiment but in no case did it exceed 2.0 μg/ml. Details of the procedure of the assay for

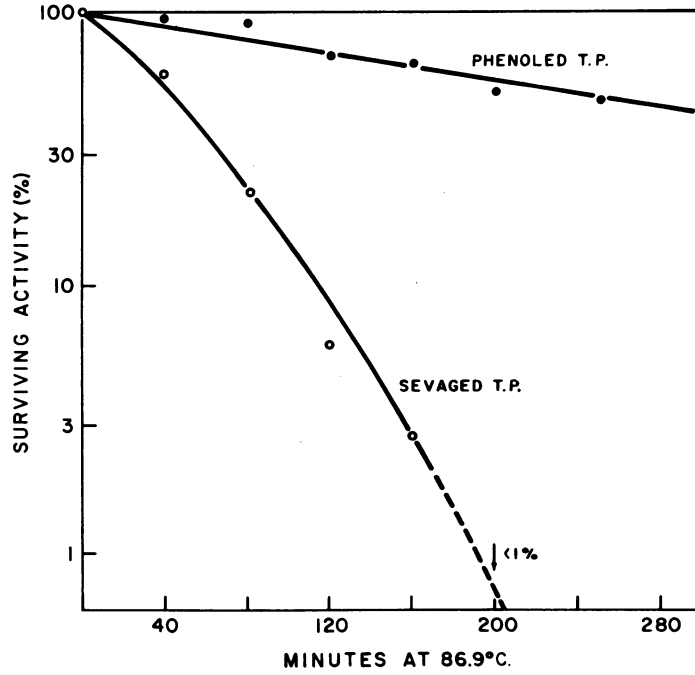

FIG. 1.—Heat inactivation of Phenoled TP and Sevaged TP at 86.9°C in 0.1 M sodium phosphate buffer, pH 7.25, at a concentration of 0.5 μg DNA/ml.

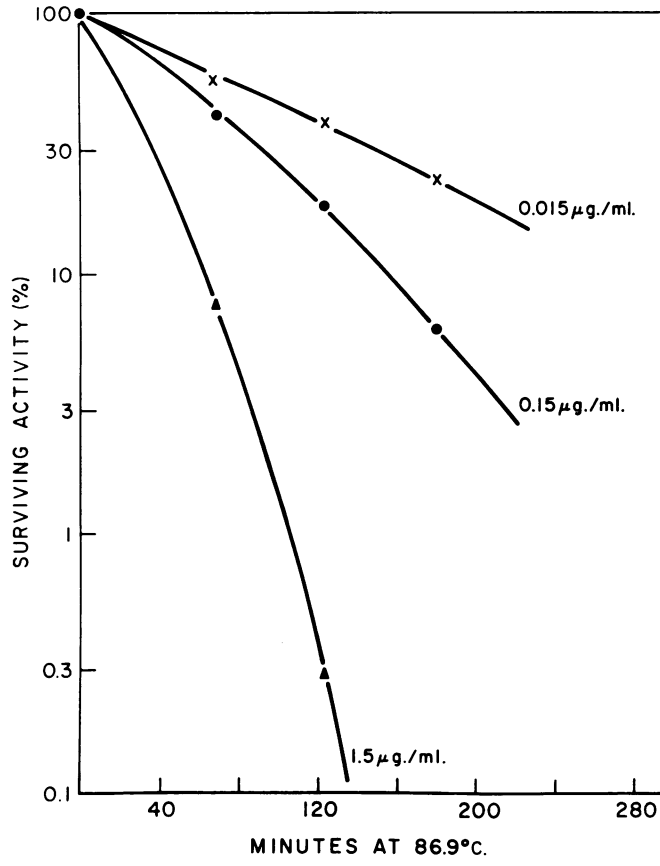

FIG. 2.—Dependence of the inactivation rate of Sevaged TP on concentration of DNA at 86.9°C in 0.1 *M* sodium phosphate buffer, pH 7.25.

transformation are given elsewhere.[8] Three-tenths ml samples, collected at various time intervals, were quick-cooled by transferring into test tubes immersed in a cold-water bath and stored in the refrigerator until assayed. All samples were assayed in a concentration range where the concentration-response was linear.

Figure 1 shows the inactivation curves of the Sevaged TP and Phenoled TP heated simultaneously in the same water bath at 86.9°, both at a concentration of 0.5 μg/ml. It shows a striking contrast in the shape of the survival curves: where the plot of the Sevaged TP is concave downward, that of the Phenoled TP is apparently exponential.

It can be shown, moreover, that the rate of inactivation of the Sevaged TP depends markedly on the concentration of DNA during heating (Fig. 2). The rate for the Phenoled TP on the other hand remains constant over a hundred-fold concentration range, 0.01–1.0 μg DNA/ml (determined in separate experiments). Three samples at 0.015, 0.15, and 1.5 μg Sevaged TP/ml were heated simultaneously in the same water bath at 86.9°. Samples collected at various time intervals were quick-cooled and all diluted to the same concentration of DNA before assaying. While the curvature at 0.015 μg/ml level of concentration has apparently dis-

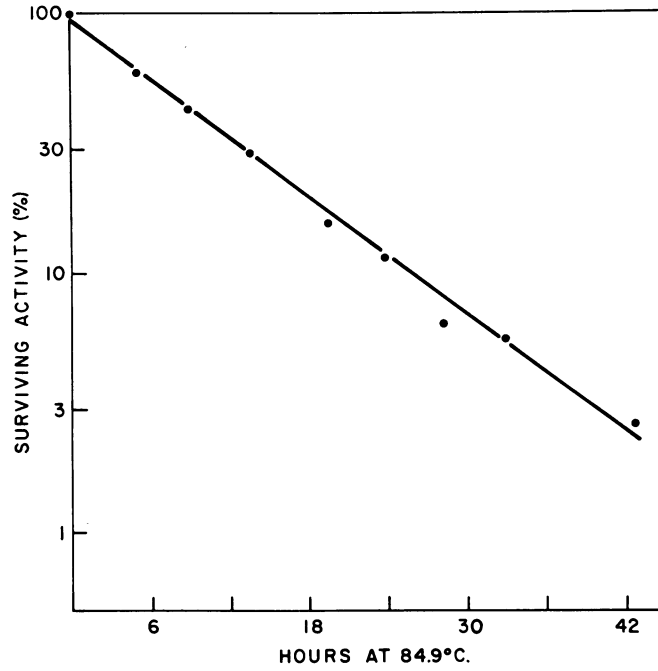

FIG. 3.—Heat inactivation of Phenoled TP at 84.9°C in 0.1 *M* sodium phosphate buffer, pH 7.25, at a concentration of 0.05 μg DNA/ml.

appeared, the rate of inactivation is still 2.3 times greater than for the Phenoled TP.

Figure 3 shows a single inactivation curve of the Phenoled TP at 84.9°. Again, the exponential nature of the inactivation is apparent over a course of 43 hours during which the survivors are reduced to about two per cent of the starting transforming activity. Also, there is no difference at this temperature between four drug-resistance markers (streptomycin, erythromycin, bryamycin, and novobiocin).[1]

Significantly, the rate of inactivation at 86.9° of III-DNA-A ($k = 4.5 \times 10^{-5}$ sec^{-1}) is very similar to that of the Phenoled TP at this temperature ($k = 4.9 \times 10^{-5}$ sec^{-1}). The plot is shown in Figure 4. All survival curves of III-DNA-A, as of Phenoled TP, at temperatures below 87° are exponential for at least 4–5 hours of heating and in the case demonstrated in Figure 4 for a 24-hour course during which 98 per cent of the initial activity is destroyed. Two-component curves become detectable at 88°, a sign of beginning of a second mode of inactivation, i.e., denaturation, which shows a sharp "melting-out" transition as the temperature is raised, with the midpoint for the streptomycin marker occurring at 88.9° for III-DNA-A and 89.4° for the Phenoled TP.[1]

Inasmuch as some residual protein or other material bound to the DNA was suspected as the cause of the complex behavior of the Sevaged TP, its density was examined in a CsCl gradient by comparison to a reference DNA of considerably higher density.[3] The densities of the Sevaged and Phenoled TP's agreed within 0.0005 gm/cm^3, which is probably below the errors of the technique (the Sevaged TP had the greater apparent density). On the other hand, the high salt concentra-

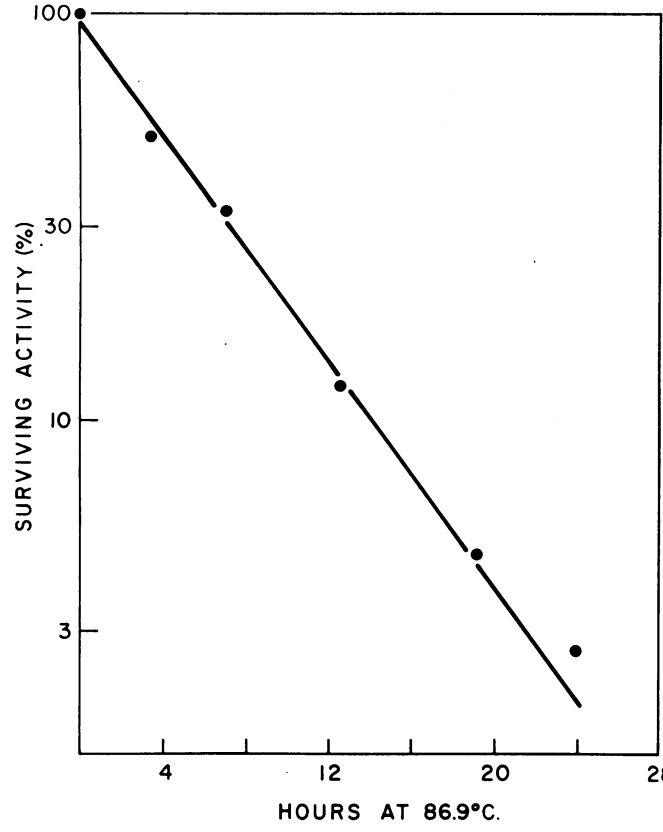

FIG. 4.—Heat inactivation of III-DNA-A at 86.9°C in 0.1 *M* sodium phosphate buffer, pH 7.25 at a concentration of 0.05 μg DNA/ml.

tion is expected to dissociate protein from DNA. From another experiment, we had a fraction of this Sevaged TP which had been collected as a single drop out of a band in a CsCl gradient, so that all unbound contaminating materials should have been removed from the DNA. Figure 5 shows the heat sensitivity of this fraction compared to the others. It is intermediate in stability, suggesting that some but not all of the material has been removed. 0.3 per cent of protein should cause a density decrease of 0.001 gm/cm^3, which was not observed. If this material is protein, the heat sensitivity of TP is a rather sensitive test for its presence.

Discussion.—Heat-induced loss of biological activity of transforming DNA has been ascribed to two mechanisms: chemical degradation (depurination and possibly phosphodiester bond cleavage) and denaturation.[1, 2, 9] At lower temperatures (<87°), where only chemical degradation is discernible, we find that DNA deproteinized by chloroform-isoamyl alcohol is markedly more heat-sensitive than the same DNA further deproteinized by water-saturated phenol. Moreover, in the latter case, the kinetics of inactivation are apparently first-order and independent of DNA concentration, whereas they are complex in the former. The rate dependence on concentration seen in chloroform-isoamyl alcohol–treated DNA suggests a higher-order inactivation process superimposed on the "one-hit" process

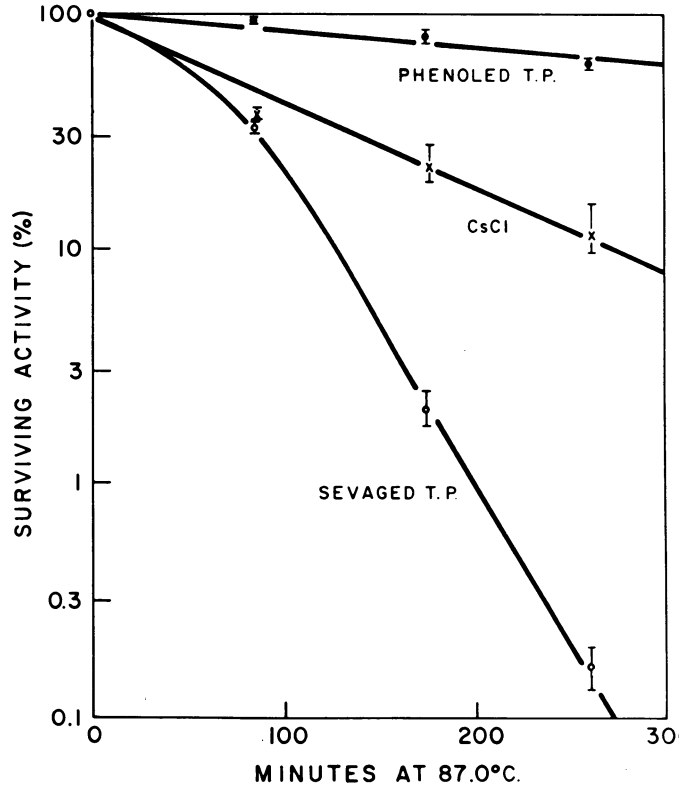

FIG. 5.—Comparative heat sensitivity at 87°C of Phenoled TP, Sevaged TP exposed to CsCl, and Sevaged TP in 0.1 *M* phosphate buffer at pH 7.25.

seen in the phenol-deproteinized material.

The most probable cause of this behavior would be a heat-induced aggregation process due to the presence of an extraneous material, for which protein is the likely candidate. Phenol is thought to be a more efficient deproteinizing agent than chloroform-isoamyl alcohol, whose use must be repeated many times to obtain minimum protein levels. For many purposes in transformation studies, it had not been obvious till now that it was important to carry the purification to the furthest limit, and a few cycles of "sevaging" sufficed. Auxiliary evidence that the degree of protein removal is the key comes from Lerman and Tolmach's work,[2] where the sevag procedure was repeated till no scum remained at the interface. Their curves are reasonably exponential, although their DNA appears to be less stable than our Phenoled TP. The possibility of another material has not been ruled out, but whatever it is, it must be readily removed by phenol and resistant to dialysis and CsCl-gradient separation.

There are numerous other variables in the study of heat inactivation of TP. We have made preliminary investigations of some of these. There is little or no effect of using saline-citrate vs. phosphate buffer as solvent at subcritical temperatures and no very large effect on the melting temperature, T_m. The T_m's also are not greatly affected by the sevag versus phenol procedure, though there may be

small effects here. We find no difference in the sensitivity of four markers at subcritical temperatures, whereas the T_m's are different, as first reported by Roger and Hotchkiss[4] and by Marmur and Doty.[3] No striking effect has yet been found for dependence of subcritical sensitivity on molecular weight, but there is a dependence of the T_m's on molecular weight, which varies for different markers. This will be the subject of a future report.

Summary.—The sensitivity of the transforming activity of a DNA preparation to heating at temperatures below the critical melting temperature depends strongly on the method of preparing the DNA. DNA deproteinized by a few cycles of shaking with chloroform-isoamyl alcohol showed concave-downward, concentration-dependent, survival curves. The same DNA, treated further with a single phenol extraction, exhibited exponential survival curves, which were concentration-independent and more stable and were very similar to the previously observed curves for a phenol preparation from a different strain of pneumococcus. A fraction of the first preparation recovered from a CsCl gradient showed intermediate stability.

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MECHANISMS OF INACTIVATION OF DEOXYRIBONUCLEIC ACIDS BY HEAT

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There has been much interest lately in the structure of the deoxyribonucleic acid (DNA) molecule and the effects of various agents on that structure. In particular, the effect of heat on the properties,[1] structure,[2] and biological activity[3-6] has been the subject of several investigations. Heat is especially interesting in that it leads