(SV_{40})[14-16] and measles virus.[17] Probably other viruses will be found to have similar effects on cells.

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*THE DENATURATION AND THE RENATURATION OF THE DNA OF POLYOMA VIRUS**

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Polyoma virus induces neoplastic growths in some rodents and causes cytopathic effects in mouse embryo tissue cultures.[1] It contains DNA corresponding to a molecular weight of 7.5×10^6 per intact virus particle.[2] The genetic information for the replication of the virus and for the initiation of tumors in hamsters is contained in a subviral infective agent (SIA) which can be extracted from the virus[3, 4] with phenol. The SIA consists essentially of the viral DNA which is mainly base-paired.[5] From the buoyant density (1.709 gm cm^{-3}) in CsCl gradients and from the melting profile it can be estimated that the DNA of polyoma virus should contain about 49 mol % of guanine-cytosine. The presence of small numbers of unusual nucleotides or of small amounts of constituents other than DNA could not be excluded. Sedimentation velocity studies of phenol extracts from purified

polyoma virus by the newly developed band centrifugation[5a] showed the presence of three discrete fractions.

It will be shown in this paper that the DNA of polyoma virus exhibits some properties which have not been reported for other base-paired, functional DNA.

Material and Methods.—The SIA and the DNA from purified preparations of polyoma virus[6] were extracted with phenol in the presence of Na-trichloroacetate (pH 8.0) and EDTA according to a technique described previously.[4] The infective titer of phenol extracts was determined by a modified plaque assay method.[4]

The treatment of the phenol extracts with enzymes and with formaldehyde, and the methods used for the preparative and analytical sedimentation equilibrium centrifugation in CsCl density gradients, will be described elsewhere.[5] DNA from bacteriophage λ (b_2b_5c) was extracted with phenol and assayed for infectivity according to a method described by Kaiser.[9]

Thermal denaturation and thermal reannealing: Thermal denaturation and reannealing were performed essentially according to Marmur and Lane[7] and Marmur and Doty.[8] The buffers in which the extracts were heated at 100°C were either 0.1 *M* NaCl-0.05 *M* tris pH 8.0. 0.1 *M*

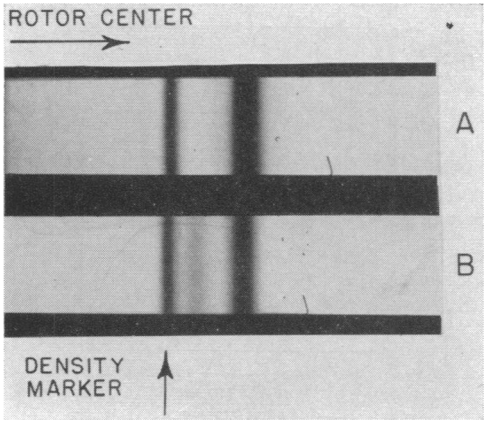

FIG. 1.—The behavior during sedimentation equilibrium centrifugation in CsCl of polyoma DNA freshly extracted from purified virus (DNA from *M. lysodeikticus*[13] used as a density marker, ρ = 1.732 gm cm^{-3}). (*A*) Native polyoma DNA. (*B*) Polyoma DNA (3γ/ml) heated for 5 min at 98.7°C in 0.01 *M* citrate-phosphate buffer, pH 7.0; quenched in ice water.

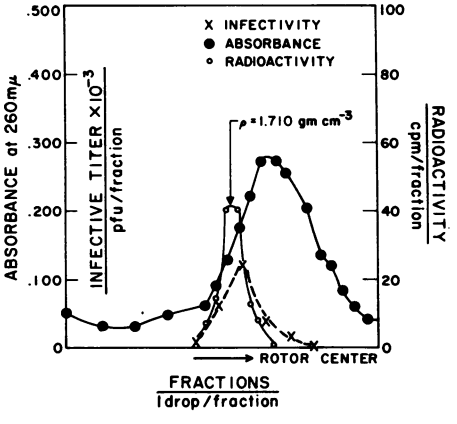

FIG. 2.—*Native polyoma SIA:* Untreated phenol extract from infected mouse embryo cell cultures. *E. coli* C^{14}-DNA added as a density marker (ρ = 1.710 gm cm^{-3}). Centrifuged in CsCl gradient at 35,000 rpm for 43 hr. Pfu = plaque-forming units.

NaCl-0.02 *M* citrate-phosphate, or 0.02 or 0.001 *M* citrate-phosphate, pH 7.0. The rapid cooling was done either by immersing the glass vials into ice water or by diluting the heated samples at the end of the heating period with 10 to 20 volumes of an ice-cold buffer. Thermal reannealing was done by adjusting the heat-denatured phenol extracts to 0.3–0.4 *M* NaCl (pH 7.0), followed by heating for 15 to 30 min at 70°C.

Denaturation and reannealing by formamide: For the denaturation of DNA the procedures of Helmkamp and Ts'o,[10] and Marmur and Ts'o[11] were followed. Formamide (99%, Matheson, Coleman, and Bell) of low conductivity and neutral pH were used at concentrations ranging from 85–99% v/v. In all the experiments the final concentration of NaCl was below 0.01 *M*. The results were essentially the same whether the incubation in formamide had been carried out at room temperature (23°C) or at 50°C. The formamide was removed by dialyzing the samples at 4°C against buffers containing 0.1 *M* NaCl. In some experiments the reannealing procedure developed by Studier[12] was used: the denatured DNA preparation was reannealed for 2 hr at room temperature in 50% v/v formamide, in the presence of 0.5 *M* NaCl (neutral pH) and thereafter the formamide dialyzed out.

Results.—The DNA extracted from purified preparations of polyoma virus formed in the CsCl gradient in the analytical ultracentrifuge a single, rather broad band with the peak at a density of 1.709 gm cm^{-3} (Fig. 1*A*). Phenol extracts from infected mouse embryo tissue cultures were centrifuged under comparable conditions in the preparative ultracentrifuge. The mouse DNA formed a broad band. The peak of the band containing the infectivity of native phenol extracts nearly coincided with that of the radioactive *E. coli* DNA (1.710 gm cm^{-3})[13] used as a density marker (Fig. 2).

The denaturation of polyoma DNA: (*a*) *Thermal denaturation:* Polyoma DNA has a relatively sharp melting profile with the midpoint, T_m, at about 89°C (0.15 *M* NaCl). The transition was complete within a few minutes at 95°C as judged by the optical absorbance at 260 mμ. After heating and rapid cooling under

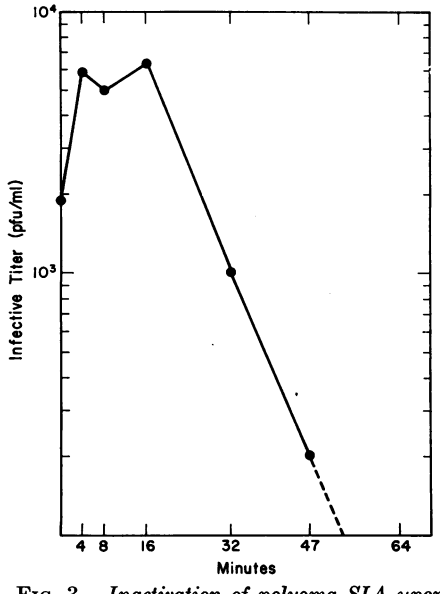

FIG. 3.—*Inactivation of polyoma SIA upon heating at 100°C:* Phenol extract heated at 100°C for the indicated lengths of time in 0.1 *M* NaCl-0.01 *M* tris pH 8.0. Aliquots cooled in ice water and immediately assayed for infectivity.

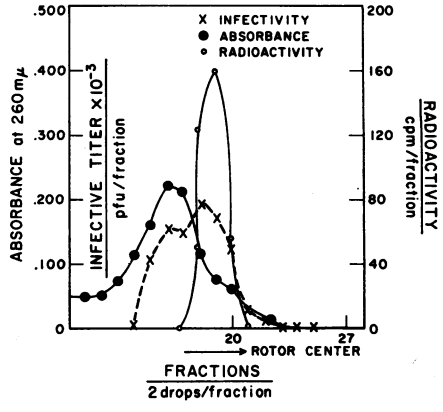

FIG. 4.—*Heated and rapidly cooled polyoma SIA:* Aliquot of the phenol extract shown in Fig. 1 heated at 100°C for 10 min in 0.1 *M* NaCl-0.05 *M* tris pH 8.0. Cooled in ice water. Native *E. coli* C^{14}-DNA added prior to density gradient centrifugation.

conditions sufficient to melt polyoma DNA, the infective titer of phenol extracts containing the SIA regularly increased by a factor of about three. The effect was independent of the amount of heterologous DNA present (at least up to 300 γ/ml) and of the ionic strength within the range tested. The same increase was observed when the SIA had been subjected to density gradient centrifugation or when it had been incubated with chymotrypsin prior to the heating. In contrast, the DNA of bacteriophage λ, heated under the same conditions, lost about 99 per cent of the original infectivity. Slow cooling as well as reannealing at 70°C resulted in a marked decrease of the infectivity of phenol extracts containing the SIA with respect to rapidly cooled controls.

Upon heating at 100°C, followed by rapid cooling, the infectivity of phenol extracts was remarkably stable up to about 15 min (Fig. 3).

In the CsCl density gradient the infective material contained in phenol extracts which had been heated for 5 to 10 min at 100°C (followed by rapid cooling) formed two bands with about the same amounts of infectivity. The lighter band corresponded to the density of largely renatured, and the heavier band to the density of denatured, polyoma DNA (Fig. 4). The bimodal distribution was the same whether the heating at 100°C had been done in 0.1 *M* NaCl or in 0.001 *M* citrate-phosphate. It is likely that clusters made up entirely of the base-pair guanine-cytosine would have melted out in 0.001 *M* citrate-phosphate.[14] The assumption that the observed increase in the buoyant density of a fraction of the SIA was caused by the collapse of its secondary structure was further supported by the high sensitivity to formaldehyde[15] of the SIA in the band of higher buoyant density (Fig. 5). Native SIA showed a very low sensitivity to 1.8 per cent formaldehyde, (at 37°C, pH 7.0, about one "lethal hit" within 18 hours). With polyoma DNA freshly extracted from purified virus similar results were obtained upon heating at 100°C followed by quenching in ice water. The DNA formed two distinct bands in a CsCl gradient in the analytical ultracentrifuge. One band had the buoyant density of denatured, and the other that of largely renatured, polyoma DNA (Fig. 1*B*).

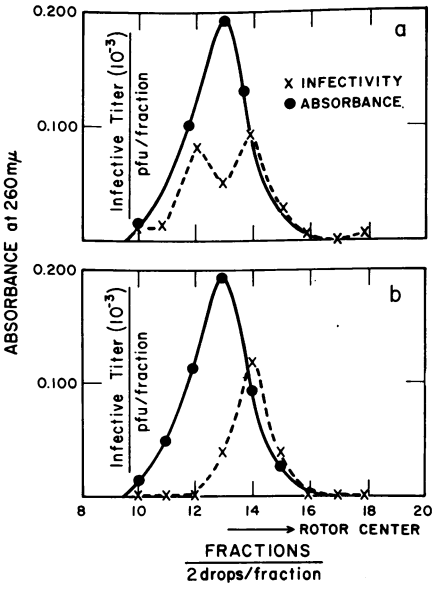

FIG. 5.—*Sensitivity to formaldehyde of heated and rapidly cooled polyoma SIA after CsCl density gradient centrifugation:* Phenol extract heated for 6 min at 100°C in 0.1 *M* NaCl-0.01 *M* tris pH 8.0, quenched in ice water, and centrifuged in a CsCl density gradient for 44 hr. Fractions collected and divided in two aliquots. Both aliquots incubated at 45°C for 40 min (pH 7.0); aliquot B in the presence of 1.8% formaldehyde. Samples dialyzed for 2 days prior to the assay for infectivity.

The DNA of bacteriophage λ was fully denatured after heating for 1 min at 100°C (followed by rapid cooling) as determined by the increase of the buoyant density in a CsCl gradient.

(*b*) *Denaturation by formamide:* Incubation with formamide under conditions which were adequate to denature and to inactivate preparations of λ DNA to about 1 per cent of the native control, did not result in a decrease of the infective titer of phenol extracts containing the SIA. Incubation in formamide in the presence of 1.8 per cent formaldehyde at neutral pH and room temperature resulted in a rapid and complete loss of the infectivity. In CsCl density gradients the banding pattern of the formamide-treated SIA was not significantly different from that of untreated controls (Fig. 6). The mouse DNA present in the treated phenol extracts, however, showed the expected increase in the buoyant density and the loss of the characteristic melting profile. It is likely that the SIA denatured during the treatment with formamide but that it renatured while the reagent was dialyzed out.

(*c*) *Denaturation under alkali and acid conditions:* Polyoma DNA denatured at 25°C around pH 12 and showed in CsCl density gradients the expected marked

shift toward a higher buoyant density.[16] Phenol extracts containing the SIA did not show any detectable loss of infectivity upon incubation at 37°C at pH 7.0 in 0.03 *M* citrate-phosphate buffer for at least 30 hr. Incubation at a pH of 3 or below resulted in a very markedly increased rate of inactivation. The relatively slow onset of the inactivation upon lowering the pH to 3 or below suggests that the inactivation was probably not due to the collapse of the helical structure *per se*.[17]

The renaturation of polyoma DNA: If phenol extracts were heated in 0.02 *M* citrate-phosphate at 100°C for 5 to 10 min and rapidly cooled, then incubated with 1.8 per cent formaldehyde, pH 7.0 at 37°C, about 95 per cent of the heated SIA was more than 200 times as sensitive to the reagent as the unheated SIA. About 5 per cent of the heated and rapidly cooled SIA showed a sensitivity to formaldehyde which was comparable to the low sensitivity of native SIA. The heated and rapidly cooled SIA remained equally sensitive to formaldehyde if it was stored in 0.02 *M* citrate-phosphate pH 7.0 for 24 hr at 4°C prior to the incubation with the reagent.

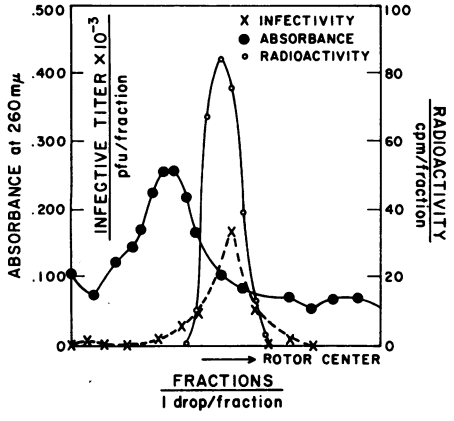

FIG. 6.—*Formamide-treated polyoma SIA:* Phenol extract incubated for 25 min at 37°C in 84% (v/v) formamide (0.01 *M* NaCl-0.005 *M* tris pH 8.0-0.001 *M* EDTA); thereafter dialyzed at 4°C against 0.1 *M* NaCl-0.05 *M* tris pH 8.0-0.001 *M* EDTA. Native *E. coli* C^{14}-DNA added prior to density gradient centrifugation.

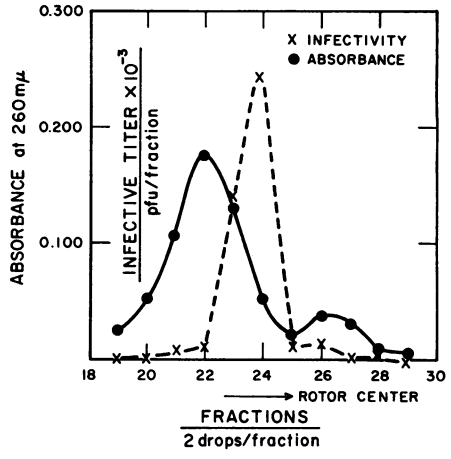

FIG. 7.—*Thermal reannealing of heated and rapidly cooled polyoma SIA:* Phenol extract heated at 100°C for 10 min in 0.1 *M* NaCl-0.01 *M* tris pH 8.0. Cooled in ice water. Reannealed for 30 min at 70°C in 0.32 *M* NaCl-0.03 *M* tris pH 8.0.

However, addition of 1.8 per cent formaldehyde immediately after the heating and cooling had little effect on the extent to which (purified) polyoma DNA renatured spontaneously, as determined by its buoyant density. Thus, it is probable that the reformation of the helical configuration took place during or soon after the cooling. The marked sensitivity to formaldehyde of most of the heated and rapidly cooled SIA, as detected by loss of infectivity, suggests that the arrangement of the complementary strands on most of the reformed helices was not fully regular immediately after the rapid cooling, and that a more regular arrangement took place in the CsCl solution (Figs. 4 and 5).

Preliminary experiments in the analytical ultracentrifuge showed that the extent to which purified polyoma DNA renatures spontaneously after heating and rapid cooling decreases as a function of the time of heating at elevated temperatures.

Similarly, "aged" (4°C) polyoma DNA showed a decreased tendency to renature spontaneously.

The renaturation of heat-denatured SIA occurred readily following incubation in formamide. Dialyzing the reagent out was sufficient for renaturation to occur. Similarly, thermal reannealing of heat-denatured SIA (Fig. 7) or (fully) heat-denatured purified polyoma DNA restored the buoyant density of the SIA and of the DNA nearly to that of the native controls. After thermal reannealing the SIA lost its high sensitivity to formaldehyde. Attempts to reanneal formamide-denatured preparations of λDNA (2 μg/ml) with formamide did result in a very small increase in the infectivity only.

Discussion.—The DNA of polyoma virus shows some properties which have not been reported for any other base-paired, functional DNA. Thus, the collapse of the secondary structure does not impair its biological activity. The differences of the effect of heating on polyoma DNA and transforming bacterial DNA can be explained, at least partially, by the properties of the assay systems used in the two cases.

The uptake of native as well as of denatured polyoma DNA by mouse embryo cells proceeds in at least two distinct steps:[18] a rapid initial attachment to the cells is followed, under adequate experimental conditions, by a time and temperature dependent process which possibly corresponds to the uptake of the DNA into the cells. Radioactive polyoma DNA which had been heated for 5 or for 20 min at 100°C (followed by rapid cooling) attached to mouse embryo cell monolayers 3 to 5 times more efficiently than the unheated DNA. Thus, the regularly observed increase in the infective titer of phenol extracts after heating under conditions where polyoma DNA melted out, at least temporarily, can possibly be explained by a more efficient initial attachment of the heated SIA to the mouse embryo cells. On the contrary, the uptake by intact bacterial cells of transforming DNA is sharply reduced upon denaturation.[19]

The stability of the infective titer upon heating at 100°C is not understood. The loss of infectivity upon reannealing for 30 min at 70°C stands in apparent contrast to the stability of the SIA when heated at 100°C (followed by rapid cooling). The reason for this is not known; it may be due partially to a reversal of the phenomenon leading to the increase in the infectivity after heating and rapid cooling.

The outstanding feature of the DNA of polyoma virus is its great readiness to renature. Because of its homogeneity, the renaturation of the DNA extracted from purified polyoma virus preparations is expected to occur readily under optimal reannealing conditions.[8] However, no other functional, helical DNA has been reported so far which shows a comparable tendency to renature spontaneously after heating and rapid cooling in buffers of relatively low ionic strength as does the DNA of polyoma virus. This behavior is somewhat reminiscent of that of helical DNA, the strands of which have been cross-linked by treatment with nitrous acid, bifunctional nitrogen mustard,[20] or by irradiation with ultraviolet light.[21] However, on the basis of the evidence presented, a complete separation of the complementary strands followed by a very rapid nucleation and reformation of the helical structure cannot be excluded.

The DNA's of two other mammalian viruses, adenovirus (type 2)[22] and pseudorabies virus,[23] with molecular weights similar to that of polyoma virus, show a

melting behavior which is in accordance with that reported for other base-paired DNA.

The biological significance of the above-mentioned properties of polyoma DNA is not known. The similarity in several respects of polyoma virus with at least two other tumor-producing viruses, the Shope papilloma virus[24] and the virus SV 40,[25] is remarkable. They are the only mammalian viruses so far reported to yield an "infective DNA" upon extraction with phenol.[26] The "infective DNA" of all three viruses shows a comparable thermal stability.[27, 28] The physico-chemical properties of the DNA of polyoma virus are similar in some respects to those of the DNA of Shope papilloma virus.[29] Preliminary experiments, however, suggest that the buoyant density of the DNA of the three viruses is not identical. A better understanding of the nature of the DNA of polyoma virus and that of related mammalian viruses has to be acquired in order to learn whether there might exist a causative relation between the observed properties of polyoma DNA and its oncogenic potential.

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AN APPROXIMATE CELL MODEL FOR LIQUID HYDROGEN, II

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Communicated by Henry Eyring, January 29, 1963

In a previous publication[1] (hereafter called I), an approximate cell model was formulated and applied to liquid hydrogen. In this approximate model the potential well of Lennard-Jones and Devonshire[2] was approximated by a parabolic well split by a region of constant potential. This approximate cell model appears to preserve the essential features of the Lennard-Jones and Devonshire theory but does not involve the computational difficulties of the exact method of Levelt and Hurst.[3]

In this publication the variation of thermodynamic properties among the H_2 isotopes is reported.

Comparison of Tabulated Properties and Conclusions.—In Table 1 the reduced internal entropy is tabulated for the five H_2 isotopes. The reduced thermodynamic functions have been defined in I and the potential parameters ($\epsilon/k = 37.00°K$, $\sigma = 2.928$ Å) have been assumed to be the same for all five liquids. The appropriate values of the quantum parameters of these five liquids, obtained from Hirschfelder *et al.*,[4] have also been included in Table 1. The communal entropy correction has been introduced and, therefore, the internal entropy tends to zero at high temperatures and low densities. In Tables 2 and 3 the reduced internal energy and the reduced internal specific heat are tabulated. The internal energy and specific heat are in reasonable agreement with the calculations of Levelt and Hurst.[3] The agreement of the internal entropy with the calculations of Levelt and Hurst is less satisfactory.

As was reported in I, this approximate cell model does not give satisfactory values for volume derivatives. However, it does give reasonable results for the quantum correction. If the quantum correction to the reduced pressure is added to the reduced pressures, calculated by Hirschfelder *et al.*,[4] for the classical cell theory, a good estimate of the critical constants may be obtained. The calculated and experimental critical constants of the five H_2 isotopes are given in Table 4. The experimental critical constants for H_2, HD and D_2 were obtained by Hoge and Lassiter.[5] The "experimental" critical constants of DT and T_2 are given in parentheses, since they are estimates given by Friedman *et al.*[6] based on an assumed linear variation of the critical properties with λ^*. For comparison, the calculated and experimental critical constants for a classical liquid have also been included. The calculated