*EFFECT OF X IRRADIATION ON THE PRIMING ACTIVITY OF DNA**

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Communicated by Harland G. Wood, November 26, 1963

The recent experiments of Opara-Kubinska, Lorkiewicz, and Szybalski have implicated alteration of the DNA molecule to be a primary cause of the failure of cell reproduction following irradiation.[1] However, the amount of irradiation necessary to inhibit cell division (50–500 r) has usually not led to detectable changes in the chemical, physical, and biological properties of DNA.[2] The present experiments indicate that the priming activity of DNA for the RNA- and DNA-polymerases of *E. coli* is severely depressed by doses of irradiation of 1,000 r and less. The irradiation was found to be effective whether delivered to whole cells or to dilute solutions of DNA.

Materials and Methods.—DNA: DNA was isolated by the methods of Hurst,[3] Kay, Simmons, and Dounce[4] (0.15 *M* NaCl–0.015 *M* sodium citrate was used in place of H_2O for dissolving the DNA), or Marmur.[5] The source of the DNA was either calf thymus or leukemic lymphoblasts (L 5178 Y) grown in suspended culture. (Calf thymus DNA, prepared by the method of Hurst,[3] was the generous gift of Dr. George Becking, Department of Biochemistry, Western Reserve University. The leukemic lymphoblast, L 5178 Y, was originally obtained from Dr. Glenn Fischer, Department of Pharmacology, Yale University. The medium used was S103[6] supplemented with the following components: biotin, L-glutamine, L-isoleucine, L-asparagine, L-serine, and folic acid to give a final concentration in gm/l of 1×10^{-5}, 0.2, 0.15, 0.01, 0.04, and 0.01, respectively. Horse serum was also added to 20% v/v.)

Polymerases: The RNA- and DNA-polymerases of *E. coli* were separated and partially purified either by the method of Hurwitz *et al.*[7] or by the method of Aposhian and Kornberg.[8] In the initial experiments the enzymes in the high-speed supernatant fraction were precipitated with protamine, eluted, and separated by fractionation with ammonium sulfate.[7] (We are indebted to Dr. George Becking for his aid in the preparation of the partially purified enzyme.) Preparations used at this stage of purification will be designated as "HC." In later experiments DNA-polymerase was further purified by chromatography on DEAE, followed either by two additional ammonium sulfate fractionations[7] or by chromatography on phosphocellulose.[8] These two enzyme preparations will be designated as "HP" and "AK VII," respectively.

Assay: The amount of polynucleotide formed was determined according to Hurwitz *et al.*[7] and Furth *et al.*,[9] by measuring the conversion of labeled nucleoside triphosphate into acid-insoluble material in an incubation medium consisting of 4 nucleoside triphosphates (one of which was labeled), Mn^{++}, Mg^{++}, tris buffer, mercaptoethanol, DNA primer, and the enzyme preparation. Since very little conversion of labeled triphosphate into the acid-insoluble form occurred in the absence of DNA, the assay served as a measure of the efficacy of the DNA as primer for polynucleotide synthesis.

Irradiation: DNA was irradiated either *in vivo* (in the intact cell) or *in vitro* (in dilute solution). For the *in vivo* irradiation, approximately 2×10^9 lymphoblasts were harvested, suspended in 10–20 ml of growth medium, and exposed to 1,000 r at the rate of 73 r per minute (250 kv, 15 ma, filtered through 1.5 mm Cu). The cells were centrifuged and frozen immediately after the irradiation, the DNA being extracted at a later date. For *in vitro* irradiation, DNA from lymphoblasts or from calf thymus was dissolved in 0.01 *M* phosphate buffer, pH 7.0, to give a final concentration of 30–100 μg/ml, and the solution was irradiated as described above. Unless specified otherwise, the radiation dose was 1,000 r. Control suspensions of cells or solutions of DNA were treated similarly except that the irradiation was omitted.

Radioactive nucleoside triphosphates: dATP-C^{14} and ATP-H^3 were obtained from Schwarz BioResearch,Inc. Radioactivity was measured with a Nuclear-Chicago liquid scintillation counter. Aqueous samples were counted in a scintillant consisting of 12.5% naphthalene, 0.75% 2,5-di-

phenyl oxazole (PPO), and 0.0375% 1,4-bis-2(4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP) in dioxane. (Naphthalene and dioxane are Eastman white label reagents. PPO and dimethyl POPOP were obtained from Packard Instrument Company, Inc.)

Protein was determined by the method of Lowry *et al.*[10] In most cases the protein was first precipitated with trichloracetic acid in order to eliminate substances interfering with the color reaction.

Results.—Irradiation of DNA in dilute solution markedly reduced its priming activity for both RNA- and DNA-polymerase (Table 1). Thus, irradiation with

TABLE 1

EFFECT OF *in vitro* X IRRADIATION ON THE PRIMING ACTIVITY OF CALF THYMUS DNA

	mμmoles Labeled Nucleoside Triphosphate Incorporated into Acid-Insoluble Fraction in 20′ at 37°C		
	RNA-polymerase	DNA-polymerase	
X-ray dose (r)		(1)	(2)
0	0.079	0.610	0.945
500			0.410
1,000	0.046	0.161	0.259
10,000	0.019	0.017	
No DNA	0.007	0.002	
No enzyme	0.003	0.019	

Incubation mixtures contained: for RNA-polymerase—ATP-H^3 (20.4 μM, 2,800 cpm/mμmole), CTP, GTP, UTP (each at 40.8 μM), $MnCl_2$ (1 mM), $MgCl_2$ (4 mM), tris buffer pH 7.4 (25.4 mM), mercaptoethanol (1 mM), DNA (50 μg, unheated), PO_4 buffer pH 7 (5.4 mM), enzyme: HC-RNA-polymerase (0.025 ml, 10 μg protein) in a final volume of 0.925 ml; for DNA-polymerase–dATP-C^{14} (4.3 μM; 7,200 cpm/mμmole), dGTP, dCTP, dTTP (each at 8.6 μM), $MgCl_2$ (2.4 mM), tris buffer pH 8.4 (30.8 mM), mercaptoethanol (1.2 mM), DNA (50 μg, unheated), PO_4 buffer pH 7 (5.7 mM), enzyme: HC-DNA-polymerase (0.025 ml, 12.9 μg protein) in a final volume of 0.875 ml.

1,000 r reduced the priming activity of DNA in the RNA-polymerase reaction to 58 per cent of the control and in the DNA-polymerase reaction to 26–27 per cent of the control. Very little polymerization occurred in either system in the absence of primer or in the absence of enzyme. Less inhibition of priming activity for DNA-polymerase was observed if solutions containing more than 100 μg DNA/ml were irradiated (Table 2). Preincubation of the DNA with the enzyme and Mg^{++} led to an increased incorporation of dATP-C^{14} into acid-insoluble material upon the subsequent addition of the triphosphates. The preincubation appeared to have a greater effect in the case of the irradiated DNA than for the control DNA, so that the difference between control and irradiated primers decreased after preincubation (Table 3). The priming activity of irradiated DNA appeared to decrease when DNA-polymerase preparations of higher purity were used in the reaction mixture. Dose-response curves for the priming activity in the DNA-polymerase reaction using a crude and a more purified DNA-polymerase are shown in Figure 1.

TABLE 2

EFFECT OF DNA CONCENTRATION DURING IRRADIATION ON INHIBITION OF PRIMING ACTIVITY BY 1,000 r

Concentration of DNA during irradiation (γ/ml)	% Control priming activity for DNA-polymerase
100	50
500	62
1,000	66
4,000	86

The incubation was carried out under the same conditions as described in Table 1. After irradiation, the DNA solutions were diluted, 50 μg DNA being added to each incubation tube. The enzyme used was HC-DNA polymerase (0.01 ml, 26.5 μg protein).

Using the more purified DNA-polymerase, the time course of the reaction was determined with control and irradiated DNA as primer. As shown in Figure 2, the rate of the reaction was greatly decreased in the case of the irradiated primer, and the decrease was apparent within the first 5 min. The effect of increasing the enzyme concentration using control and irradiated primer is shown in Figure 3.

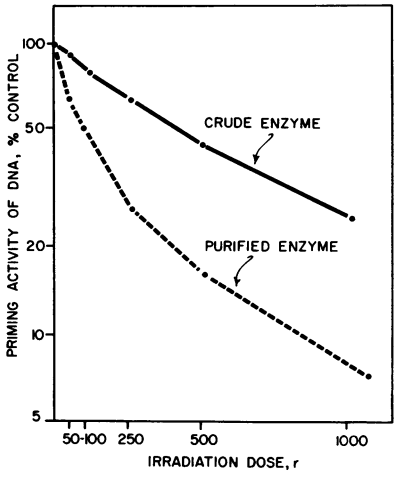

FIG. 1.—Effect of irradiation on the priming activity of DNA for crude and purified DNA-polymerase. The incubation was carried out under the conditions for DNA-polymerase described under Table 1. Solid line: enzyme = HC-DNA-polymerase, 0.02 ml, 10.3 μg protein. DNA = 50 μg/tube. Dashed line: enzyme = AK VII, 0.02 5.6 μg protein. DNA = 25 μg/tube.

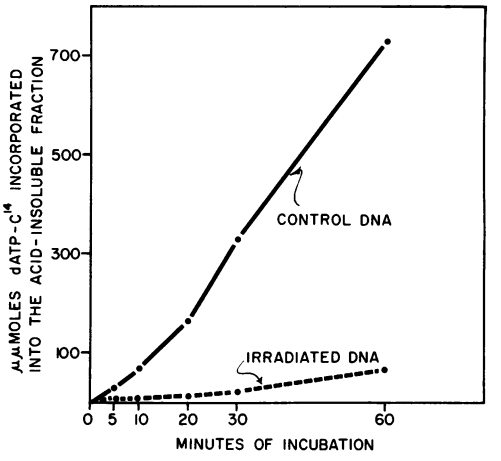

FIG. 2.—Time course of dATP-C^{14} incorporation using control and irradiated primer. The incubation was carried out under the conditions for DNA-polymerase described under Table 1. Enzyme = AK VII, 0.02 ml, 5.6 μg protein. DNA = 50 μg/tube.

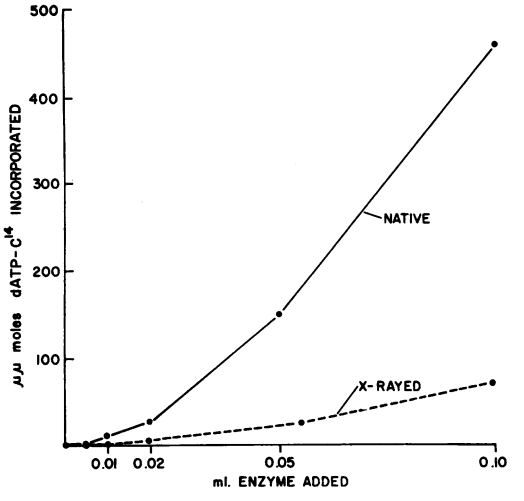

FIG. 3.—Effect of enzyme concentration on the incorporation of dATP-C^{14} using control and irradiated primer. The incubation was carried out under the conditions for DNA-polymerase described under Table 1. Enzyme = HP, 373 μg/protein/ml. DNA = 50 μg/tube.

In this experiment the polymerization in the presence of irradiated primer was about 15 per cent of that obtained with control primer at all levels of enzyme. The upward curvature observed when the incorporation of dATP-C^{14} was plotted against either time (Fig. 2) or enzyme concentration (Fig. 3) might be explained if conver-

TABLE 3

EFFECT OF PREINCUBATION OF DNA AND DNA-POLYMERASE ON SUBSEQUENT INCORPORATION OF dATP-C^{14} INTO ACID-INSOLUBLE PRODUCT

Preincubation (min)	mμmoles dATP-C^{14} incorporated		$\frac{\text{Irradiated}}{\text{Control}} \times 100$
	Control DNA	Irradiated DNA	
0	0.437	0.216	49.5
5	0.500	0.261	52.3
10	0.557	0.319	57.3
15	0.589	0.423	72.0
20	0.604	0.425	70.5

A solution of 100 μg calf thymus DNA/ml of 0.01 *M* phosphate buffer was divided in 2 parts, and half was given 1,000 r. The preincubation conditions were as follows: 50 μg DNA, phosphate buffer pH 7 (89 mM), $MgCl_2$ (3.5 mM), and 0.02 ml HC-DNA-polymerase (53 μg protein) in a final volume of 0.57 ml incubated at 37°. At the specified time nucleoside triphosphates, tris buffer pH 8.4, and mercaptoethanol were added to give the incubation conditions for polymerization described in Table 1.

sion of the double-stranded DNA to "active primer" were the rate-limiting step at early times or at low enzyme concentrations. In agreement with this suggestion, it has been found that a 5′ preincubation of DNA, Mg^{++}, and the polymerase preparation eliminated the upward curvature at early intervals. Further study of the kinetics of the reaction is in progress.

When the concentration of DNA was increased, the rate of polymerization increased to an optimum and then decreased with further increase in the amount of DNA (Fig. 4). The optimum activity was reached at a lower DNA concentration (5 μg/tube) in the case of the DNA irradiated in dilute solution than in the case of the control DNA (25 μg/tube). The amount of dATP-C^{14} incorporated into acid-insoluble material in 20′ at optimum DNA concentration was found to be 534 μμmoles for unirradiated DNA and only 140 μμmoles for DNA irradiated in dilute solution. Figure 4 also shows the activity of DNA extracted from cells given 1,000 r. The optimum DNA concentration in this case was 10 μg/tube, at which concentration 360 μμmoles of dATP-C^{14} were incorporated in 20′. Thus, DNA irradiated *in vivo* also showed reduced priming activity when used with a purified DNA-polymerase. In previous experiments, in which a crude enzyme preparation was used, no reduction in priming activity was noted for DNA extracted from irradiated cells.[11]

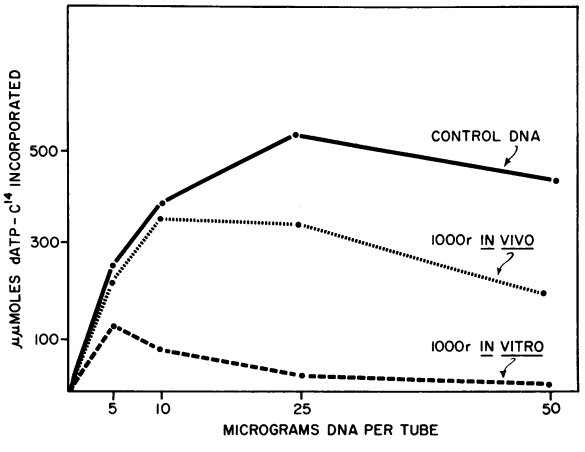

FIG. 4.—Effect of DNA concentration on the incorporation of dATP-C^{14}. The incubation was carried out under the conditions for DNA polymerase described under Table 1. Enzyme = AK VII, 0.02 ml, 5.6 μg protein.

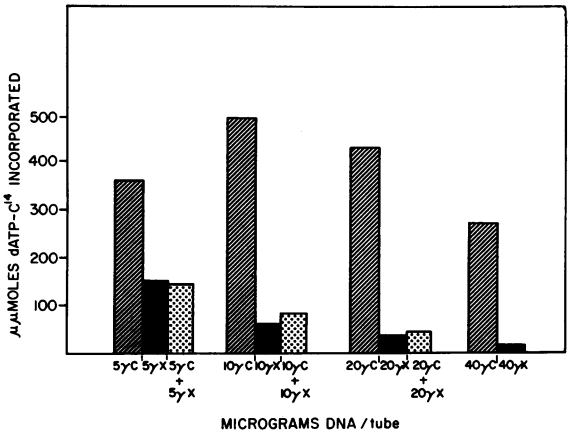

FIG. 5.—Priming activity of combinations of control and irradiated DNA. The incubation was carried out under the conditions for DNA polymerase described under Table 1. Enzyme = AK VII, 0.02 ml, 5.6 μg/protein. Diagonals: control DNA only. Solid: irradiated DNA only. Dots: equal mixtures of control and irradiated DNA.

When equal quantities of control and irradiated DNA were mixed, the priming activity of the mixture was very similar to that obtained for the irradiated DNA alone (Fig. 5). Decreasing the amount of irradiated DNA in the presence of a constant amount of control DNA resulted in greater activity, as shown in Figure 6, so that when the ratio of control DNA to irradiated DNA was 20/1, the activity of the mixture was 80 per cent of the control alone. Table 4 shows the results obtained when a constant mixture of control DNA and irradiated DNA (10/1) was incubated with increasing amounts of enzyme. When the amount of enzyme was increased 5-fold, the activity with control DNA alone or irradiated DNA alone increased approximately 6-fold, while the activity of the mixture increased 7-fold. However, even with this increase in enzyme concentration, the activity of the mix-

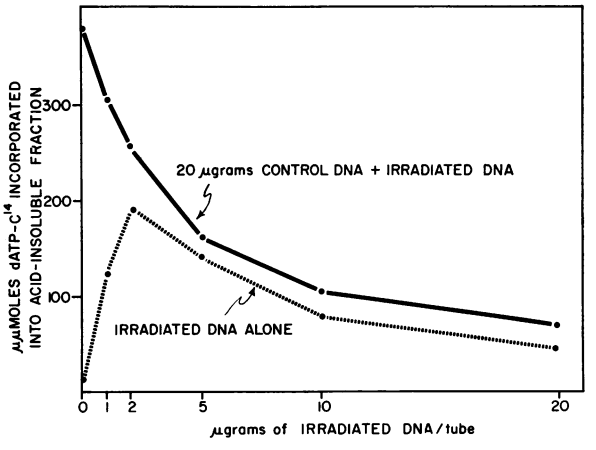

FIG. 6.—Priming activity of combinations of control and irradiated DNA, with varying amounts of irradiated DNA. The incubation was carried out under the conditions for DNA-polymerase described under Table 1. Enzyme AK VII, 0.02 ml, 5.6 μg protein.

TABLE 4

THE PRIMING ACTIVITY OF MIXTURES OF CONTROL AND IRRADIATED DNA

DNA added	Enzyme added (ml)	μμmoles dATP-C^{14} incorporated
10 μg Control	0.02	116
11 μg Control	0.02	104
1 μg Irradiated	0.02	54
10 μg Control + 1 μg irradiated	0.02	87
10 μg Control	0.05	347
11 μg Control	0.05	363
1 μg Irradiated	0.05	169
10 μg Control + 1 μg irradiated	0.05	306
10 μg Control	0.10	660
11 μg Control	0.10	656
1 μg Irradiated	0.10	300
10 μg Control + 1 μg irradiated	0.10	639

The incubation was carried out under the conditions for DNA polymerase described under Table 1. Enzyme = AK VIIa, 99 μg protein/ml.

ture was still lower than that obtained with the control DNA alone.

Discussion.—Our observation that 50–1,000 r X irradiation inhibits the priming activity of DNA is in contrast to results obtained by others, including Wheeler and Okada,[12] Stacey,[13] and Walwick and Main.[14] This discrepancy in results could be due to the different source of the polymerase (*E. coli* rather than calf thymus or regenerating liver), the purity of the enzyme preparation, the concentration of DNA during irradiation, or the use of native rather than heated DNA as primer.

The results which we have obtained indicate that the irradiation produces an alteration in the structure of the DNA molecule. Such an alteration could be a direct or an indirect cause of the reducing priming activity of DNA for the polymerization reaction. As a possible indirect action, the alteration might enable nucleases present in the polymerase preparation to enact a rapid digestion of the DNA, producing fragments too small to serve as efficient primers for the *E. coli* polymerase. However, since the inhibition increased as the enzyme was purified and the nucleases were removed, and since preincubation of DNA with the polymerase preparation increased rather than decreased the priming activity, it appears that the reduced priming activity is not caused by a more extensive degradation of the irradiated DNA by nucleases present in the polymerase preparation. (Digestion of labeled DNA by the polymerase preparation, as measured by the appearance of radioactivity in the acid-soluble fraction after a regular 20-min incubation, was 94 per cent for HC and about 5 per cent for HP and AK VII.) Alternatively, the alteration in the DNA molecule could be the direct cause of the reduced priming activity. The nature of such an alteration is as yet undetermined. Keir[15] has recently shown that a DNA polymerase preparation from Landshutz ascites tumor cells was inhibited by addition to the complete reaction mixture of oligonucleotides consisting of approximately 6 nucleotide units and ending with a 3′-phosphate. Oligonucleotides ending with a 5′-phosphate were found to be stimulatory. Although we have found no evidence of extensive degradation of DNA after X irradiation under the conditions described above, our observations of viscosity (± heat) and of the thermal transition of DNA irradiated with 1,000 r *in vivo* and *in vitro* have led to the suggestion that DNA extracted from cells irradiated with 1,000 r contains single-strand scissions,[16] whereas DNA irradiated in dilute solution contains a greater number of breaks which may be accompanied by localized denaturation.[17] Thus, the possibility exists that breaks producing a 3′-phosphate end group might cause inhibition of the polymerase reaction.

From observations on viscosity, weight average molecular weight, and the radius of gyration, Alexander *et al.* have suggested that branching and cross-linking may occur in DNA after irradiation in the solid state.[18] Bollum and Setlow[19] have found

that UV-irradiation of DNA markedly reduces its priming activity for the DNA-polymerase of calf thymus, and have suggested that the UV-induced dimers between adjacent thymine residues in the polynucleotide chain are the major photoproducts responsible for the loss in priming activity. One model which they have suggested assumes that normal replication occurs along a DNA chain until the TT dimer is reached. At this point the rate of synthesis drops to the rate for "end addition" until a loop which can bridge the dimer is formed, after which normal replication again resumes. According to this model, a mathematical function relating the rate of synthesis with dose was derived which had the same general shape as the dose-response curve which they observed. The nonexponential character of these UV-dose-response curves is very similar to those which we have obtained with X irradiation, and might be an indication that X irradiation also produces alterations in the chain which block the assembly of nucleotides along the template.

The decrease in activity observed when small amounts of irradiated DNA were added to control DNA (Figs. 5, 6) could be due to a competition between the two primers for the enzyme, the irradiated DNA having a higher affinity but a lower activity. It is hoped that further study of the kinetics of the reaction will provide information as to the mechanism of this inhibition.

Summary.—The activity of DNA as a primer for RNA- and DNA-polymerase from *E. coli* was severely depressed by X irradiation in doses of 1,000 r or less. The priming activity for DNA-polymerase appeared to be more readily affected and was reduced in the case of DNA extracted from irradiated lymphoblasts, as well as for DNA irradiated in dilute solution. The optimum concentration of primer in the DNA-polymerase reaction was lower for irradiated DNA than for control DNA, concentrations higher than the optimum being inhibitory in both cases. Mixtures of irradiated and control DNA showed a lower priming activity than that found for the control DNA alone, the extent of the decrease depending on the ratio of the concentration of the control to the irradiated DNA in the mixture, as well as on the amount of enzyme present. The nature of the alteration in the DNA caused by irradiation which is responsible for the reduced priming activity remains to be determined.

* A preliminary report of these experiments was presented at the 145th meeting of the American Chemical Society, New York, 1963. This investigation was supported by contract W31-109-eng-78 of the U. S. Atomic Energy Commission with Western Reserve University, and by research grant HD-00669-04 from the National Institute of Child Health and Human Development, U.S. Public Health Service.

† With the technical assistance of Marlene Ricanati.

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*TRANSFORMATION OF PROPERTIES OF AN ESTABLISHED CELL LINE BY SV40 AND POLYOMA VIRUS**

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Communicated by Leo Szilard, November 27, 1963

The ability of polyoma virus[1-6] and SV40[7-12] to alter permanently the properties of cultured fibroblasts has already been described in a variety of systems. We have developed an established cell line—3T3[13] possessing properties particularly well-suited to the *in vitro* analysis of transformation by these viruses. The line was developed from disaggregated Swiss mouse embryos by successive transfer of the cells every 3 days at an inoculation density sufficiently low to ensure that the cells did not attain confluence between transfers. This procedure was continued for the 30 transfers required to establish the cell line. If now the cells are allowed to reach confluence, growth is arrested when the monolayer is complete, and no growth occurs out of the plane of the monolayer; this line which, like other established lines, has unlimited growth potential and a high plating efficiency may be described as having an unusually high degree of contact inhibition of cell division. In addition, it differs from other mouse fibroblast lines evolved under somewhat different conditions in its virtually complete inability to synthesize collagen.[14] 3T3 cells are readily transformed both by polyoma virus and by SV40, the transformed cells being easily recognized as well-defined colonies of multilayered growth against the monolayer background of untransformed cells.

In most respects, the transformed cells produced by the two viruses behave similarly—both types lose susceptibility to contact inhibition and become able to grow to a high saturation density, and both become able to synthesize collagen. In certain other respects, however, the nature of the transformed cells appears different depending on the virus causing the transformation.

Materials and Methods.—Cell culture: All cultures were maintained in 50 mm plastic Petri dishes in Dulbecco and Vogt's modification of Eagle's medium[15] supplemented with 10% calf serum. The media of all cultures were changed three times weekly. The 3T3 cells used in these experiments, though established as a line under the conditions previously described, are now maintained by serial transfer of 1:1,000 dilutions of trypsinized confluent cultures.