VIRAL DNA IN POLYOMA- AND SV40-TRANSFORMED CELL LINES*

By Heiner Westphal† and Renato Dulbecco

THE SALK INSTITUTE FOR BIOLOGICAL STUDIES, SAN DIEGO, CALIFORNIA

It is clear that cells transformed by the small DNA-containing viruses, polyoma virus (Py) and SV40, harbor the viral DNA since they contain viral mRNA,[1] and some lines even yield infectious virus after fusion with cells able to support the multiplication of the virus.[2, 3] Very little is known, however, about the state of the viral DNA in these cells. In the past, several attempts were directed at determining the average number of copies of the viral DNA per cell by molecular hybridization of the DNA extracted from transformed cells with either viral DNA, or RNA made *in vitro* by copying the viral DNA. These efforts, however, were handicapped by two main technical difficulties. One is to prepare viral DNA uncontaminated by cellular DNA; the other difficulty is to have available a hybridization procedure capable of approaching the detection of one viral DNA molecule per cell, that is, one part of viral DNA among 2×10^6 parts of cellular DNA. In the experiments to be described below, both requirements have been met. Pure viral DNA was obtained by isolating the supercoiled component I[4-6] which is uncontaminated by cellular DNA. The hybridization procedure between cellular DNA and RNA made *in vitro* on purified component I DNA was that of Gillespie and Spiegelman,[7] with a number of small improvements which have increased its sensitivity and reproducibility. Fewer than five viral DNA molecules per cell can now be unambiguously identified.

In order to estimate the number of viral DNA copies per cell, it must be known whether complete molecules or only a part of them are present. This ambiguity does not exist in experiments employing the cell line SV3T3-47, from which infectious SV40 is recovered by fusion. For the other lines in which such recovery was impossible, the ambiguity persists. For this reason, we shall express the results in viral DNA equivalents per cell on the basis of reconstruction experiments.

Materials and Methods.—Viruses: Strains, purification, and DNA extraction of polyoma[8-10] and SV40[11] have been published elsewhere. The DNA extracted from the viruses was sedimented in a CsCl gradient (1.5 gm per ml 0.02 M Tris-HCl, pH 8.0, 0.002 M EDTA). The tube was centrifuged for 22 hr at 24,000 rpm (Spinco model L, SW25.1 head, 20°C); then fractions were collected from the bottom and the OD was determined. The fractions containing component I were pooled.

Cellular DNA: Mouse embryo DNA was isolated by a method previously used for solid tumors.[12] Nuclei and cytoplasm were prepared[13] from crowded tissue cultures, and the DNA was extracted with sodium dodecyl sulfate (SDS)-chloroform.[14] RNase treatment was carried out using 20 μg/ml of the enzyme for 30 min at 37°C. All DNA species were stored at 4°C with small amounts of chloroform added. The amounts of DNA were determined by the modified diphenylamine reaction.[15]

Enzymes: Purified DNA-dependent RNA polymerase (*E. coli* B) was the kind gift of Dr. Paul Berg. The enzyme was stored, without noticeable loss of activity during 6 months, in 50% glycerol at −20°C. The amounts of enzyme are given as amounts of

1158

protein of this preparation determined by Lowry's method.[16] DNase (RNase-free batch) was obtained from Worthington Biochemical, pancreatic RNase (5× crystallized) from Sigma Chemical; it was preheated at 80°C for 10 min at pH 5.0 to remove DNase traces and was then reneutralized. RNase T1 was obtained from Calbiochem.

In vitro synthesis of virus-complementary RNA (cRNA) was carried out according to Chamberlin and Berg.[17] The standard reaction mixture contained, in 400 μliters, 30 μg DNA-dependent RNA polymerase, 5 μg of SV40- or polyoma-DNA component I, 20 mμmoles each of tritiated cytidine 5'-triphosphate (CTP), 1.25 c/mmole; guanosine 5'-triphosphate (GTP), 1.0 c/mmole; uridine triphosphate (UTP), 2.3 c/mmole (all purchased from Schwarz BioResearch); and adenosine 5'-triphosphate (ATP), 8 c/mmole (purchased from the New England Nuclear Corp.). After an incubation time of 5 hr, the reaction was stopped by adding 20 μg DNase (15 min 37°C), and then SDS at 0.5% final concentration. One mg yeast-RNA (Worthington Biochemical) in 0.1 ml 0.1 × SSC was added. Then the mixture was fractionated on a Sephadex G-50 medium column (diameter 0.5 cm, bed volume 3 ml, flow rate 0.3 ml per min, equilibration buffer 0.1 × SSC, 0.1% SDS). The first peak to elute was collected, twice extracted with chloroform, dialyzed for 24 hr at 4°C against 0.1 × SSC, 0.01% SDS, and stored in small portions at −70°C. The calculation of the amount of cRNA formed was based on the assumption that the enzyme preparation does not contain nucleoside triphosphates, and that the rate of incorporation of each nucleoside triphosphate was equal. On this basis, the yield of a reaction mixture was, on the average, 20 μg cRNA, with a specific radioactivity of 5×10^6 cpm/μg (using a Beckman scintillation counter). The RNA had a sedimentation coefficient of *ca.* 4S (determined by sucrose gradient sedimentation together with marker HeLa ribosomal RNA) and contained 35–40% of the entire input of radioactivity.

DNA/RNA hybridization: The method of hybridizing RNA with DNA immobilized on nitrocellulose membranes[7] was used with the following modifications (unmodified steps are not described). Unless otherwise described, all DNA preparations were treated with DNase[1] to convert the component I of the viral DNA into a heat-denaturable form. DNA (100 μg) was treated with 8×10^{-4} μg DNase from the same batch, stabilized with 8 μg bovine serum albumin (fraction V) (Armour Pharmaceutical), in 1 ml of 0.01 M Tris-HCl, pH 8.0, containing 0.02 M MgCl₂ and 0.04 M NaCl, for 60 min at 30°C. Under these conditions, more than 90% of SV40 component I DNA was converted into component II, as shown by analytical ultracentrifugation. The DNase-treated DNA was subsequently poured into 2 vol boiling H₂O, boiled 15 min, chilled in ice, adjusted to 6 × SSC (final concentration), and slowly passed through Millipore membrane filters (HAWP 2400). Prior to use, these filters had been washed with boiling 6 × SSC in a Petri dish and then repeatedly with cold 6 × SSC on the filtration apparatus. The filters were incubated for 22 hr at 66°C in vials containing, in 1 ml 6 × SSC, tritiated cRNA, 1 mg yeast RNA, and SDS at a final concentration of 0.1%. The addition of SDS did not interfere with the specific RNA/DNA hybridization, but markedly reduced the background counts. The proportion of counts present in the input cRNA that remained on an empty filter under these conditions was 10^{-4}. During incubation, the vials were slowly shaken in a water bath. After the hybridization the filters were treated with 20 μg/ml pancreas RNase and 10 units/ml RNase T1 60 min 37°C.

After being counted in a toluene-scintillation mixture, the filters were assayed for DNA content by the diphenylamine reaction.[12] The amount of input cRNA was estimated in each case by placing 20 μliters of the hybridization mixture on a filter containing immobilized DNA, without filtering it through, but letting it dry in air. The dried filter was then counted. This procedure eliminated the need for a quenching correction.

Results.—Optimal conditions for RNA/DNA hybridization (Fig. 1): The conditions for hybridizing the viral RNA synthesized *in vitro* with cellular DNA were optimized in reconstruction experiments. These employed mixtures of viral DNA and a large excess of carrier DNA from nontransformed cells, in proportions similar to those to be expected in transformed cells. The filters
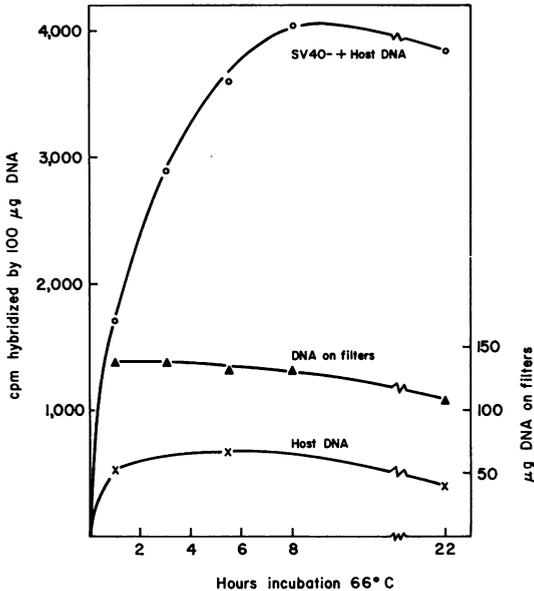
FIG. 1.—Kinetics of DNA/cRNA hybridization. Py3T3-6 DNA (which does not contain SV40-specific sequences) was mixed with SV40 DNA at a ratio corresponding to 100 viral DNA molecules per host cell, and hybridized with RNA complementary to SV40 component I DNA for the indicated periods of time. The amount of DNA immobilized per filter was 160 μg. The input of tritiated cRNA per filter was 2×10^5 cpm. The hybrid cpm/filter were normalized to an average of 100 μg DNA per filter (*see* legend, Table 3). Each measurement represents the average of five replicate filters. $\times - \times$, cpm bound to filters with carrier DNA only; $O - O$, cpm bound to filters containing the virus/host DNA mixture; $\blacktriangle - \blacktriangle$, amount of DNA on the filters, determined after counting.

contained 5×10^{-3} μg SV40 DNA per 100 μg of 3T3 DNA, corresponding to 100 viral DNA molecules per cell (Table 1). The number of counts immobilized on a filter reached a maximum after eight hours incubation at 66°C. A continuation of the incubation beyond this time caused a noticeable reduction of the amount of unspecific binding of cRNA to the carrier DNA. Although there was also a slight loss of DNA from the filters and of cRNA counts from filters containing viral DNA, an incubation time of 22 hours optimized the ratio of specific to nonspecific binding and was therefore adopted in all experiments. Under these conditions, the amount of specific hybrid counts increased linearly with the input of cRNA over a large range. Approximately 3×10^6 cpm cRNA were needed to saturate 10^{-4} μg of polyoma DNA mixed with 100 μg of carrier DNA (Fig. 2). About 1000 cpm were bound, corresponding to 2×10^{-4} μg of RNA. The precise quantitative significance of this result cannot be evaluated owing to some uncertainty of the true specific activity of the RNA. Anyway, the result shows that most sequences of the viral DNA have been copied *in vitro*.

Of the total DNA applied to a filter, a considerable proportion (up to 60%) was lost during immobilization and incubation at high temperature. Presum-

TABLE 1. *Quantitative data related to hybridization experiments.*

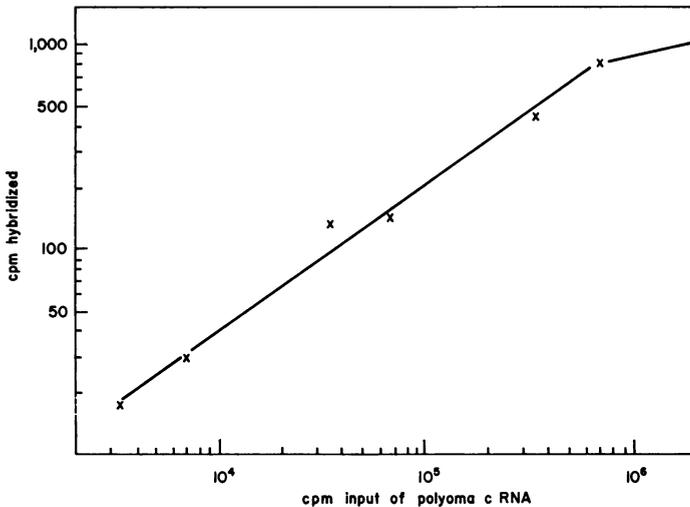|	
Weight of the DNA of a cell nucleus	10^{-5} μg
Weight of the DNA of a cell cytoplasm	10^{-8} μg
Weight of one viral DNA molecule	5×10^{-12} μg
Proportion of viral DNA in a cell containing a single molecule of DNA	5×10^{-7}
Amount of viral DNA, in 100 μg of total DNA, corresponding to 1 viral DNA equivalent per cell	5×10^{-5} μg
Tritiated complementary RNA: specific activity	5×10^6 cpm/μg

FIG. 2.—Saturation of polyoma DNA with homologous cRNA. Polyoma DNA was immobilized on nitrocellulose filters together with mouse embryo DNA (200 μg per filter) at a ratio of five viral DNA molecules per host cell (calculation, Table 1), and hybridized with given amounts of polyoma cRNA under standard conditions. The counts per minute specifically hybridized are obtained after subtracting the counts per minute bound to filters containing equal amounts of mouse embryo DNA alone. The DNA content of the counted filters was, on the average, 65 μg. Each measurement represents the average of three replicate filters (standard deviation <10%).

ably this loss occurs whenever the amounts of DNA employed were near the capacity of the filters. Thus the question was whether there was any selective loss of viral DNA molecules owing to their small size. Reconstruction experiments, however, showed that the number of hybridized counts was a function of the ratio of viral to host DNA in the original hybridization mixture, irrespective of the amount of DNA that was lost during hybridization; therefore, loss is not selective.

Virus DNA equivalents in DNA extracted from nuclei and cytoplasm of various SV40- and/or polyoma-transformed cell lines: A description of the cell lines investigated in this work is given in Table 2. No infectious virus could be isolated from the pure cultures; by cell fusion with BSC-1 cells, infectious SV40 could be isolated only from two lines; no line yielded infectious plaque virus on fusion with mouse embryo cells (Watkins and Dulbecco[3]; Dulbecco, unpublished). During several years of maintenance in different laboratories, most of the lines have been transferred through many generations.

In order to determine whether viral DNA could be demonstrated, the DNA of each line was hybridized to viral cRNA in several replicate filters. The results were compared to controls in which an identical amount of DNA from the corresponding untransformed cells was hybridized with the same RNA, using the same number of filters. The determination of the number of viral DNA equivalents per cell is based on the comparison between the number of counts specifically

TABLE 2. *Cell lines investigated.*

Cell line	Reference and description	Parent cell line	Transf. agent	Virus Rescue		T-Antigen
				Direct methods	Cell fusion	
3T3	*Todaro, Green*[18] Normal established line	3T3	−	−	−	−
SV3T3-47	*Todaro, Green*[19] SV40-transformed derivative of 3T3	3T3	SV40	−	+(SV)	+
SV3T3-56	Clone of 3T3 cells transformed by nitrous acid-treated virus	3T3	SV40	−	−	+
Py3T3-6	*Benjamin*[1] Py-transformed derivative of 3T3, isolated in agar	3T3	SV40	−		+
SVPy3T3-11	*Todaro et al.*[20] SV40, Py doubly transformed	3T3	Py and SV40	−	+(SV)	+
BHK Cl.13	*Stoker, Abel*[21] Normal baby hamster kidney	BHK	−	Not tested	Not tested	−
Py8	Py transformed derivative of BHK Cl.13 isolated in agar	BHK	Py	−	−	+
H50	*Khera et al.*[22] Derivative from an SV40-induced hamster tumor	Hamster tumor	SV40	−	−	+
ME	Normal mouse embryos	−	−	Not tested	Not tested	−

hybridized, using the DNA of a transformed line, and a calibration curve obtained from the hybridization of mixtures of untransformed cell DNA with known amounts of viral DNA. Specifically hybridized counts are those fixed by the filters containing viral DNA after subtraction of counts fixed by filters containing untransformed DNA alone. There is an increase of specific hybridization as a function of the amount of transformed cell DNA bound to the filters.

Table 3 gives the results from different SV40- and/or polyoma-transformed lines and the calibration data. The average number of SV40 DNA equivalents detected in SV3T3-47 and in H50 cells are 20 and 60, respectively. For both experiments Py3T3-6 DNA was used as an additional negative control lacking SV40 sequences. The *t*-tests of the difference of the sample means and the resulting probabilities indicate the significance of the results. In order to determine the effect of culture age on the number of detectable viral DNA equivalents, cultures of SV3T3-47 cells that were actively growing and had not yet reached confluency were compared with well-crowded cultures (containing five times as many cells per culture). The number of SV40 DNA equivalents was equal in the two cases. The examination of many replicate DNA preparations per cell line, however, would be necessary to give an accurate calculation of the average number of viral DNA equivalents per transformed cell under different conditions.

Hybridization experiments were extended to several other lines transformed

TABLE 3. *SV40 DNA equivalents in SV40-transformed cell lines.*

DNA source	Cpm per 100 µg DNA	Specific Cpm*	Hybridization Probability	Viral DNA equiv./ cell
Expt. 1				
3T3 (control)	1040 ± 87 (10)†	0	—	—
3T3 + 5 molecules SV40 DNA/cell	1390 ± 115 (9)	350	<0.002	8
3T3 + 16 molecules SV40 DNA/cell	1670 ± 164 (9)	630	<0.002	16
SV3T3-47	1840 ± 115 (10)	800	<0.002	20
Py3T3-6	1135 ± 214 (10)	95	>0.5	5
Expt. 2				
3T3 (control)	745 ± 108 (5)	0	—	—
3T3 + 20 molecules SV40 DNA/cell	1120 ± 113 (5)	375	<0.002	20
SV3T3-47	1135 ± 80 (5)	390	<0.002	21
Py3T3-6	695 ± 93 (5)	−50	>0.5	—
BHK Cl.13 (control)	600 ± 68 (9)	0	—	—
BHK Cl.13 + 10 molecules SV40 DNA/cell	805 ± 85 (9)	205	<0.002	10
H50	1800 ± 198 (9)	1200	<0.002	58
Expt. 3				
3T3 (control)	483 ± 53 (5)	0	—	—
SVPy3T3-11	1428 ± 35 (5)	945	<0.002	44
SV3T3-47	908 ± 54 (5)	425	<0.002	20
SV3T3-47Y‡	886 ± 133 (5)	403	<0.002	19
Expt. 4				
3T3 (control)	715 ± 99 (12)	0	—	—
SV3T3-47	1482 ± 70 (8)	767	<0.002	20
SV3T3-56	979 ± 100 (12)	264	<0.002	7

Using the standard procedure, tritiated RNA complementary to SV40 (component I) DNA was hybridized with the indicated DNA species. The H^3 input of cRNA per filter was: expt. 1, 4 × 10^5 cpm; expt. 2 and 4, 3 × 10^5 cpm; expt. 3, 2 × 10^5 cpm. 160 µg DNA was added per filter. The amount of DNA remaining on the filters after hybridization was determined and the hybrid cpm of each filter were normalized to an average of 100 µg DNA. SV40 DNA component I was used to calibrate the system.

* Cpm after subtracting the 3T3 control values from the transformed 3T3 DNA species; after subtracting the BHK Cl.13 control from H50.

† Cpm ± standard deviation and in parentheses, the number of filters.

‡ SV3T3-47Y DNA is derived from cells harvested when semiconfluent.

by SV40 and/or polyoma virus (Tables 3 and 4).   It was found that the SVPy-3T3-11 line contains about 40 SV40 and 10 polyoma DNA equivalents per cell. The number of SV40 DNA equivalents in the line SV3T3-56 is only 7, i.e., one third of that found in SV3T3-47.   Line Py3T3-6 and the Py8 line of polyoma-transformed hamster cells contain about 5 and 7 Py DNA equivalents, respectively.   SV3T3-47 DNA and H50 DNA failed to give specific hybridization with polyoma cRNA.

When the distribution of the virus-specific DNA between nucleus and cytoplasm in transformed cells was examined, using lines Py3T3-6, SV3T3-47, and H50, all hybridizable counts were found in the nucleus (Table 5).

Discussion.—The results reported confirm the previous reports that viral DNA can be detected in cells transformed by either polyoma virus or SV40.[23, 24] Furthermore, the improved experimental technique allows for the first time a reliable estimation of the average numbers of virus DNA equivalents per cell based on reconstruction experiments.   The main result is that the number of equivalents

TABLE 4. *Polyoma DNA equivalents in Py-transformed cell lines.*

DNA source	Cpm per 100 μg DNA	Specific Cpm	Hybridization Probability	Viral DNA equiv./ cell
Expt. 1				
3T3 (control)	1400 ± 140 (10)	0	—	—
3T3 + 10 molecules Py DNA/cell	2100 ± 150 (9)	700	<0.002	10
3T3 + 20 molecules Py DNA/cell	2950 ± 320 (9)	1550	<0.002	20
Py3T3-6	1750 ± 130 (10)	350	<0.002	5
SV3T3-47	1400 ± 95 (10)	0	>0.5	—
Expt. 2				
3T3 (control)	891 ± 24 (6)	0	—	—
Py3T3-6	1100 ± 57 (6)	209	<0.002	5
SVPy3T3-11	1318 ± 68 (6)	427	<0.002	10
Expt. 3				
BHK Cl.13 (control)	530 ± 46 (9)	0	—	—
BHK Cl.13 + 7 molecules Py DNA/ cell	747 ± 45 (9)	217	<0.002	7
Py8	745 ± 39 (9)	215	<0.002	7

Tritiated RNA complementary to polyoma (component I) DNA was hybridized with the indicated DNA species. The H^3 input per filter was: expt. 1, 5×10^5 cpm; expts. 2 and 3, 2×10^5 cpm. Polyoma DNA component I was used to calibrate the system. Further details are given in the legend of Table 3.

per cell varies in different lines from about 60 (H50 line) to a number close to the limit of significant detection (Py3T3-6), and they are localized in the nucleus. In many lines, the number of equivalents is much higher than the ploidy of the cells employed.

As to the biological significance of these differences, we shall remark that lines with a higher average number of viral DNA equivalents were not "more" transformed, as judged from the morphological and growth properties of the cells in culture. An especially interesting line is SV3T3-56, which was derived from a clone transformed by nitrous acid-treated virus and does not yield infectious virus on fusion with BSC-1 cells with the help of UV Sendai virus (unpublished observations), in contrast to SV3T3-47 and SVPy3T3-11.[3] Thus it was probably transformed by a mutant virus unable to cause a productive infection. The low number of equivalents present in this line, compared with other SV40-transformed derivatives of line 3T3, suggests that the expression of a viral gene function may be required for building up a large number of DNA equivalents per cell.

There is no correlation between numbers of viral DNA equivalents per cell

TABLE 5. *Distribution of the virus-specific DNA between nucleus and cytoplasm in transformed cells.*

Cell line	No. of Viral DNA Equivalents per Nucleus	Cytoplasm
SV3T3-47	20	≤ 0.03
Py3T3-6	5	≤ 0.005
H50	60	≤ 0.04

Cytoplasmic and nuclear DNA were separately hybridized with the appropriate complementary RNA (2×10^5 cpm/filter). After counting, 8 μg DNA were, on the average, bound per filter. Calibrations and controls were like those given in Table 3.

and ability of the cells to yield infectious virus after fusion with virus-susceptible cells.   For instance, line H50, with 60 equivalents, does not yield virus on fusion. It may have a mutated viral DNA.   There may be a correlation between the number of equivalents and the amount of T-antigen formed because minimal amounts of the antigen are formed in line SV3T3-56.

An interesting question concerns the origin of the many copies of viral DNA present in the cells of certain lines.

For understanding this problem the physical characteristics of the viral DNA molecules present in the cells must be determined.   Work in this direction is in progress.

Summary.—The amounts of viral DNA present in polyoma- and SV40-transformed cell lines have been determined by hybridizing the cellular DNA with *in vitro* RNA complementary to viral DNA.   The numbers of virus DNA equivalents per cell varies from 5 to 60 in different lines.   The viral DNA is located in the nuclei of the cells.   The biological significance of these findings is discussed.

[1] Benjamin, T., *J. Mol. Biol.*, **16**, 359 (1966).
[2] Koprowski, H., F. C. Jensen, and Z. Steplewski, these PROCEEDINGS, **58**, 127 (1967).
[3] Watkins, J. F., and R. Dulbecco, these PROCEEDINGS, **58**, 1396 (1967).
[4] Dulbecco, R., and M. Vogt, these PROCEEDINGS, **50**, 236 (1963).
[5] Weil, R., and J. Vinograd, these PROCEEDINGS, **50**, 730 (1963).
[6] Crawford, L. V., and P. H. Black, *Virology*, **24**, 388 (1964).
[7] Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).
[8] Vogt, M., and R. Dulbecco, *Virology*, **16**, 41 (1962).
[9] Winocour, E., *Virology*, **19**, 158 (1963).
[10] Weil, R., *Virology*, **14**, 46 (1961).
[11] Hatanaka, M., and R. Dulbecco, these PROCEEDINGS, **56**, 736 (1966).
[12] Winocour, E., *Virology*, **25**, 276 (1965).
[13] Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell, these PROCEEDINGS, **49**, 654 (1963).
[14] Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961).
[15] Burton, K., *Biochem. J.*, **62**, 315 (1956).
[16] Lowry, O. H., A. L. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[17] Chamberlin, M., and P. Berg, these PROCEEDINGS, **48**, 81 (1962).
[18] Todaro, E. J., and H. Green, *J. Cell Biol.*, **17**, 299 (1963).
[19] Todaro, E. J., and H. Green, *Science*, **147**, 513 (1965).
[20] Todaro, E. J., K. Habel, and H. Green, *Virology*, **27**, 179 (1965).
[21] Stoker, M., and P. Abel, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 27 (1962), p. 375.
[22] Khera, K. S., A. Ashkenazi, F. Rapp, and J. L. Melnick, *J. Immunol.*, **91**, 604 (1963).
[23] Axelrod, D., K. Habel, and E. T. Bolton, *Science*, **146**, 1466 (1964).
[24] Reich, P. R., P. H. Black, and S. M. Weissman, these PROCEEDINGS, **56**, 78 (1966).