INDUCTION OF CELLULAR DNA SYNTHESIS BY POLYOMA VIRUS

By Roger Weil, Marcel R. Michel, and Gisela K. Ruschmann

DEPARTMENT OF VIROLOGY, SWISS INSTITUTE FOR EXPERIMENTAL CANCER RESEARCH, LAUSANNE, SWITZERLAND

Communicated by André Lwoff, April 9, 1965

Infection with polyoma (Py) virus can lead to synthesis of progeny virus, a reaction which prevails in mouse kidney (MK) cell cultures.[1] The other type of response, predominant in hamster embryo fibroblasts, leads to cellular transformation in the absence of viral replication.[2-4] In this preliminary report we will show that synthesis of Py virus in confluent MK cells takes place in two distinct phases and that the actual replication of viral progeny (phase 2) is accompanied by virus-induced synthesis of host DNA.

Experimental.—MK cultures were prepared according to Winocour[1] and infected with 0.2 ml of the same strain of wild-type Py virus as used in our previous work (about 5×10^8 plaque-forming units/ml). Crude lysate and virus purified by CsCl density gradient centrifugation were found to exhibit the same biological effects. MK cultures were infected 2–3 days after confluence. At this time most cells have stopped mitotic division and DNA synthesis. Confluent MK cultures contain about 3×10^6 cells, most of which (80–90%) have a polygonal shape and are virus producers. The remainder are spindle-shaped cells of which few, if any, synthesize progeny virus. They show decreased contact inhibition, continue after confluence to undergo mitosis at a slow rate, and are responsible, as shown by autoradiography, for continued synthesis of small amounts of DNA in uninfected, confluent monolayers. Combined microspectrophotometry on Feulgen-stained cells[5] (scanning UMSP I, Zeiss) and chemical determinations of total DNA[6] show that 80–90% of the uninfected cells contain a diploid, and the others a tetraploid DNA complement. The effect of Py virus is the same in kidney cells from inbred Sn-A mice (a gift of Prof. G. Klein), inbred white Swiss mice, or random-bred white mice.

For pulse-labeling experiments, deoxythymidine-H^3 ($TdRH^3$) or 5-bromodeoxyuridine-2-C^{14} ($BUdR\text{-}C^{14}$), obtained from New England Nuclear Corp., were used as indicated in the text. At the end of the pulse the monolayers were washed three times, then covered with growth medium, and incubated for 1 hr at 37°C prior to DNA extraction. Within less than 30 min, 99% of the $TdRH^3$ taken up by the cells is incorporated into cellular or viral DNA and remains cell-associated for at least 4 hr.

Purified preparations of Py virus contain 3 DNA fractions with sedimentation velocities of 20S (I), 16S (II), and 14S (III).[7-9] Fraction I accounts for 80–90% of the total and contains circular, double-stranded DNA which shows spontaneous renaturation and resistance to shear. Py DNA II exhibits the normal melting behavior of linear DNA. Newer experimental results obtained by Vinograd[10] and in our laboratory (unpublished) suggest that Py DNA II is also circular, but that it contains a discontinuity in one of the strands. Both I and II have a molecular weight of about 3×10^6 and a base composition of 49 mol% cytosine-guanine. Fraction III has a base composition of only 46 mol% CG. Extraction by a modified SDS- (sodium dodecylsulfate) method,[11] to be described elsewhere, leads to the recovery of 90% or more of the labeled mouse DNA in a form which sediments faster than Py DNA (Fig. 2). Further extraction with chloroform-isobutanol (25:1) does not alter the pattern of sedimentation. Cellular DNA can be broken into slower sedimenting fragments by homogenization under conditions where 60–70% of Py DNA I retains its circular configuration (Fig. 4).

Sedimentation velocity analyses by band-centrifugation and CsCl density gradient equilibrium centrifugation were performed according to Vinograd *et al.*[12, 13] Radioactivity was measured in a liquid scintillation counter (Nuclear-Chicago). The infective titer of Py DNA was determined by a plaque assay described earlier.[14] The methods used for cytological investigations will be given in detail elsewhere.

Results.—DNA synthesis in confluent MK monolayers after infection with Py virus at 37°C: The small rate of $TdRH^3$-incorporation by uninfected, confluent mono-

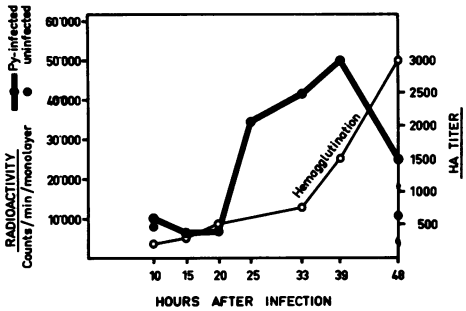

FIG. 1.—Uptake of TdRH^3 at 37°C by uninfected and by Py-infected, confluent MK monolayers. TdRH^3 5 μC/ml (spec. act. 13.8 C/mM) added to growth medium for period of 90 min at times indicated. Values are the average of 3 monolayers, each separately extracted with SDS.

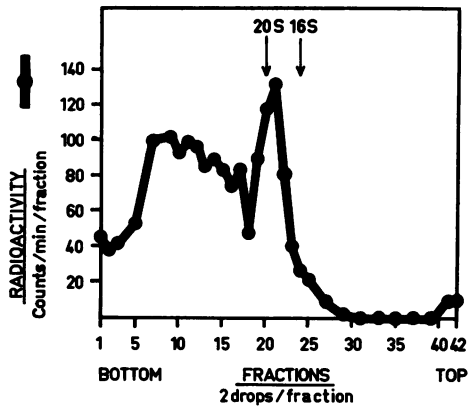

FIG. 2.—Sedimentation velocity analysis (band-centrifugation) of TdRH^3-labeled DNA from Py-infected MK monolayers, 39 hr p.i. Lamella: 0.1 ml of same material as in Fig. 1. Bulk solution: 3 ml CsCl ρ: 1.503 gm cm^{-3}, pH 7.1. Spinco model L, rotor SW 39, 35,000 rpm, 3.5 hr, 23°C.

layers remains essentially unchanged for a week or longer, while infected, confluent monolayers begin to incorporate TdRH^3 at markedly larger rates at 20–25 hr p.i. (post infectionem). The increased rate of uptake continues for about 25 hr and then falls fairly rapidly to the level of uninfected controls or below (Fig. 1). Autoradiography at 25 hr p.i. reveals that 10–15 % of the polygonal cells in infected monolayers incorporate TdRH^3 as compared to less than 0.5 per cent in uninfected controls. However, at this time only 1–3 % of the infected cells react with fluorescein-labeled antibodies directed against viral capsid protein. This suggests that DNA synthesis precedes appearance of viral capsids, a conclusion also reached by Minowada.[15] As time proceeds, the fraction of cells which synthesize viral progeny increases and reaches about 80 per cent two days p.i.

Sedimentation velocity analyses (band-centrifugation) of TdRH^3-labeled DNA extracted with SDS from infected and from uninfected monolayers do not reveal any significant differences until 16 hr p.i. Thereafter, extracts from infected monolayers show a new, well-defined band with a sedimentation velocity of 20S (Fig. 2) which contains infective Py DNA, has the buoyant density of Py DNA (1.709 gm cm^{-3}) in CsCl gradients, and is relatively stable to mechanical shear. This is Py DNA I. In most extracts a small second band corresponding to Py DNA II (16S) is also present.

Experiments in progress point to the possibility that a 16S DNA may play the role of an intermediate in the synthesis of Py DNA I (B. Hirt, unpublished). None of the extracts, however, contain labeled DNA with a sedimentation rate corresponding to that of Py DNA III. The salient feature of these results is the finding that the larger fraction (60–80%) of TdRH^3 incorporated by infected monolayers during the period of increased uptake (20–45 hr p.i.) is incorporated into *cellular* DNA. Later (>50 hr p.i.) the situation is reversed, and most of the TdRH^3 is used for synthesis of Py DNA. Analyses of TdRH^3-labeled DNA by CsCl density gradient equilibrium centrifugation support these conclusions. The decreased up-

take of TdRH[3] at later stages of infection is due to diminished synthesis of cellular DNA, to loss of infected cells, and to dilution of TdRH[3] in an increased pool of endogenous TdR (see below).

The relative amounts of Py DNA: DNA's from infected and from uninfected MK cells display essentially the same buoyant density distribution in CsCl gradients at neutral pH. Py DNA I denatures at pH 12 to form a double-stranded cyclic coil which exhibits an abnormally high density in CsCl gradients[9] and gives rise to a small band on the dense side of the mouse DNA (Fig. 3). DNA from infected MK cells can be treated in such a way that cellular DNA is denatured while Py DNA is fully renatured.[7] Equilibrium centrifugation in CsCl at pH 11 leads to separation of renatured viral from denatured cellular DNA.[16] Such experiments show that Py DNA never accounts for more than 5 per cent of the total at any time after infection and that cellular DNA has not undergone any gross changes in molecular configuration or in base composition.

Preparative band-centrifugation of phenol-extracted, sheared DNA at neutral pH leads to similar conclusions and shows that the specific radioactivity of pulse-labeled Py DNA is higher than that of mouse DNA (Fig. 4).

The physical state of MK DNA following infection with Py virus: DNA of actively growing MK cells was labeled with TdRH[3]. The monolayers were infected after confluence, and the cellular DNA was analyzed by band-centrifugation at pH 7 and 12 at different times (up to 3 days) after infection. No evidence for a breakdown of cellular DNA or for the presence of virus-induced single-strand breaks could be detected by this method. This makes it appear unlikely that increased incorporation of TdRH[3] at 20–45 hr p.i. is the reflection of a repair mechanism.[17] Other experiments show that TdRH[3] from cellular DNA is not reutilized for the synthesis of Py DNA. From a few purified preparations of Py virus grown on TdRH[3]-prelabeled MK monolayers, however, radioactive 14S DNA could be extracted which probably had been synthesized prior to infection and was en-

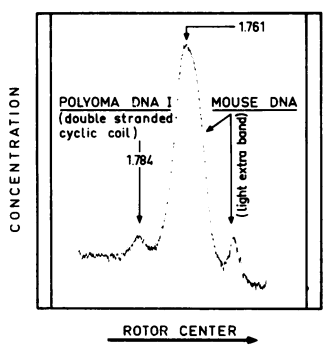

FIG. 3.—Alkaline CsCl density gradient equilibrium centrifugation of DNA from Py-infected MK cells. DNA extracted with SDS 48 hr p.i. CsCl ρ: 1.777 gm cm^{-3}, adjusted to pH 12.5 with KOH. Analytical ultracentrifuge Spinco model E, 44,770 rpm, 26 hr, 25°C. Densitometer tracing (Joyce-Loebl).

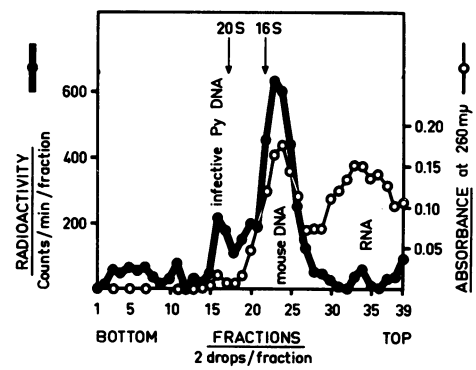

FIG. 4. — Sedimentation velocity analysis (band-centrifugation) of homogenized phenol extract from Py-infected MK cells. TdRH[3]-pulse at 48 hr p.i. as described in Fig. 1. Extracted and homogenized in presence of phenol[14] in Servall homogenizer (microattachment OM-2000), 176 V, 30 sec, 4°C. Centrifuged as described in Fig. 2.

cased in viral capsids. In view of its sedimentation velocity it cannot be linked covalently to Py DNA I or II.

Evidence for virus-induced synthesis of cellular DNA: Feulgen-microspectrophotometry suggested that cellular DNA may undergo several rounds of replication in a considerable fraction of infected cells, not followed by mitosis. The DNA content of infected monolayers determined chemically was lower than expected from these results and never exceeded that of uninfected controls by more than 40 per cent. Chemical determinations, however, give too low estimates, since morphologically "intact" infected monolayers may have lost a third or more of the

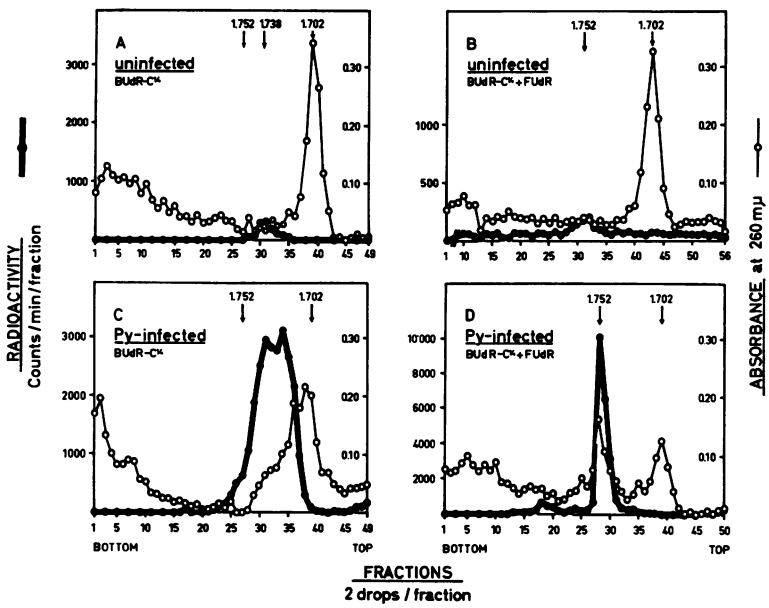

FIG. 5.—CsCl density gradient equilibrium centrifugation of BUdR-C^{14}-labeled DNA from uninfected and from Py-infected, confluent MK monolayers. (*A*) and (*B*) uninfected controls: (*A*) 4 days after confluence 8 μg/ml BUdR-C^{14} (spec. act. 19 mC/mM) added to growth medium for 24 hr; (*B*) same as (*A*), but 10^{-5} *M* FUdR in addition to BUdR-C^{14}. (*C*) and (*D*) Py-infected: (*C*) MK monolayers infected 3 days after confluence; 8 μg/ml BUdR-C^{14} present at 26–50 hr p.i. (*D*) same as (*C*), but 10^{-5} *M* FUdR in addition to BUdR-C^{14}. Monolayers extracted with SDS. 3 ml CsCl solution ρ: 1.750 gm cm^{-3}, pH 7.1, Spinco model L, rotor SW 39, 35,000 rpm, 38 hr, 23°C.

cells present prior to infection. Clear evidence for virus-induced cellular DNA synthesis could be obtained by the following experiments.

Uninfected, confluent MK monolayers incorporate little BUdR-C^{14}. Analyses in CsCl density gradients show that the bulk of cellular DNA remains unlabeled and that it has the density distribution of normal mouse DNA (1.702 gm cm^{-3}). A very small fraction of the DNA is detectable only by radioactivity bands at 1.738 gm cm^{-3} (Fig. 5*A*). It represents mainly DNA from spindle-shaped cells which underwent one cycle of semiconservative replication and where, judging from the buoyant density,[18] about 72 per cent of TdR in the newly synthesized strand is substituted by BUdR-C^{14}. The presence of FUdR (5-fluorodeoxyuridine) at a concentration sufficient to block *de novo* synthesis of TdR in mouse cells[19] leads to a

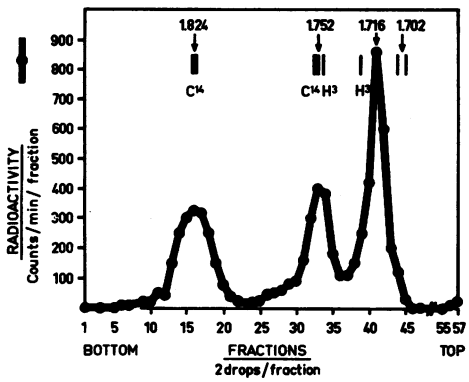

FIG. 6.—CsCl density gradient equilibrium centrifugation of heat-denatured density hybrid. Separation of the "old" strand ($TdRH^3$) from the newly synthesized strand ($BUdR\text{-}C^{14}$). DNA of actively growing MK cells labeled with $TdRH^3$. Monolayers infected with Py virus 2 days after confluence. $BUdR\text{-}C^{14}$ and FUdR added as described in Fig. 5. Main fraction of hybrid DNA (ρ: 1.752 gm cm^{-3}) dialyzed for 3 days against 10^{-3} *M* Na-phosphate, pH 8. Hybrid boiled for 3 min and cooled in ice water. Centrifuged as described in Fig. 5. In the light band (ρ:1.716 gm cm^{-3}) less than 5% of $TdRH^3$ substituted by $BUdR\text{-}C^{14}$.

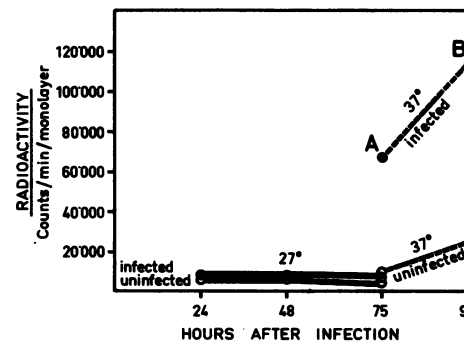

FIG. 7.—Uptake of $TdRH^3$ at 27°C by uninfected and by Py-infected, confluent MK monolayers. Effect of transfer to 37°C. Confluent MK monolayers infected and kept at 37°C for 3 hr, then incubated at 27°C. Aliquots transferred to 37°C at 71 hr p.i. $TdRH^3$-pulse: 10 μC/ml, 60 min either at 27°C or at 37°C. Band-centrifugation shows that at (*A*) about 5%, and at (*B*) about 25% of $TdRH^3$ are incorporated into Py DNA I (20S).

small increase in the uptake of $BUdR\text{-}C^{14}$ and to a shift of the radioactive band to 1.752 gm cm^{-3} (Fig. 5*B*). This radioactive DNA represents a density hybrid, where in the newly synthesized strand TdR has been fully (>95%) substituted by $BUdR\text{-}C^{14}$. The observed densities are very close to those reported by Chun and Littlefield on BU-substituted mammalian DNA.[20] The minor effect of FUdR suggests that the endogenous pool of TdR in uninfected MK cells is relatively small. The situation is different in *infected*, confluent MK monolayers where uptake of $BUdR\text{-}C^{14}$ is markedly larger than in uninfected controls. If $BUdR\text{-}C^{14}$ is added 24 hr p.i. for periods of 12–50 hr, virus-induced cytopathic effects and production of hemagglutinin, i.e., capsid proteins, are comparable to those developing in the absence of the base analogue, though little, if any, infective progeny virus is formed. CsCl density gradients reveal that most of the cellular DNA is radioactive and has shifted to higher buoyant densities (Fig. 5*C*).

Addition of FUdR to infected MK monolayers leads to a strong increase in uptake of $BUdR\text{-}C^{14}$; however, none of the virus-induced cytopathic alterations develop. In CsCl density gradients the DNA forms two bands (Fig. 5*D*). The light one corresponds to normal unsubstituted mouse DNA, while the other contains a density hybrid (1.752 gm cm^{-3}) of high specific radioactivity which accounts for 60–70 per cent of the total DNA. Thermal denaturation of the hybrid leads to physical separation into an "old" strand synthesized prior to infection containing no detectable (<5%) $BUdR\text{-}C^{14}$ and a newly synthesized strand where TdR is fully replaced by $BUdR\text{-}C^{14}$ (Fig. 6). Some of the hybrid DNA renatures spontaneously and its density remains 1.752 gm cm^{-3}. The impediments to chain separation which this material obviously contains might also be responsible for the

inability of BU-substituted mammalian DNA to undergo a second cycle of replication as reported for several cell systems.[20, 21] The effect exerted by FUdR suggests that infected MK cells contain a larger pool of newly synthesized TdR than uninfected cells.

Evidence for a psychrosensitive step in the replication of Py virus: Stimulated by the finding of a critical temperature-sensitive event in the replication of poliovirus, as reported by Lwoff,[22] we performed similar experiments. Confluent MK monolayers were infected under standard conditions, then incubated for 2–4 hr at 37°C and thereafter at 27°C for 3 days. At 27°C no evidence was obtained for synthesis of Py DNA, for induction of celluar DNA synthesis, or for presence of viral capsids detectable by electron microscopy (G. Petursson, unpublished). Though actively growing MK cells do not synthesize progeny virus at 27°C, about 25 per cent develop large vacuoles in the cytoplasm and stop further mitotic division within less than 24 hr p.i. This shows that synthesis of cellular DNA *per se* is not sufficient for viral replication. If infected, confluent monolayers are kept at 27°C for 3 days and are then transferred to 37°C, intense synthesis of cellular and viral DNA begins within <60 min after transfer (Fig. 7). This suggests that most of the preliminary steps of the eclipse phase (*phase 1*) take place at 27°C with the exception of a psychrosensitive event which initiates *phase 2*, i.e., the actual replication of progeny virus and the induction of cellular DNA synthesis. The inhibitory effect of puromycin on the replication of Py virus described by Gershon and Sachs[23] shows that during phase 1 "early proteins" are synthesized. Sheinin[19] reported that Py-infected mouse embryo fibroblasts synthesize empty viral capsids under conditions where DNA synthesis is interrupted by FUdR. Replication of Py virus thus represents a sequence of metabolic events which can be blocked at different levels.

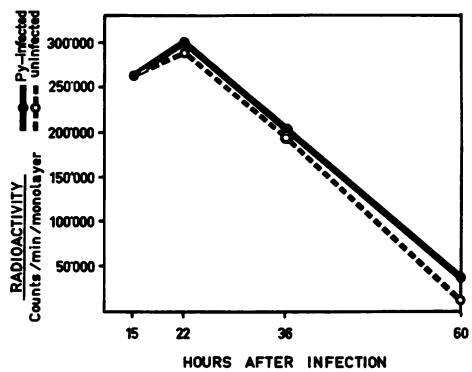

FIG. 8.—Uptake of TdRH^3 at 37°C by uninfected and by Py-infected hamster embryo fibroblasts. Secondary cultures infected one day after confluence. TdRH^3-pulse: 10 μC/ml, 60 min.

The effect of Py virus on hamster embryo fibroblasts: Hamster embryo cultures were infected prior to or shortly after confluence. In accordance with others,[2–4] we found that only very few cells synthesize progeny virus. However, soon after infection, up to 30 per cent exhibit marked nuclear fragmentation which can be prevented by actinomycin D (unpublished). Three days after confluence, infected cultures incorporate about three times more TdRH^3 than uninfected controls (Fig. 8). Autoradiography reveals that at this time 3 per cent of the infected and 1 per cent of the uninfected cells synthesize DNA. Analyses by band-centrifugation show that the TdRH^3 is incorporated into cellular DNA and that labeled Py DNA—if present—is below the level of detection. We assume that the slightly increased synthesis of DNA in infected cultures reflects virus-induced synthesis of

cellular DNA. We are presently studying whether this induction can take place in hamster fibroblasts without replication of progeny virus.

Discussion.—Replication of Py virus in confluent MK cells at 37°C proceeds in two distinct phases. Phase 1 represents a sequence of metabolic events which precede and initiate the actual replication of progeny virus. It corresponds essentially to the eclipse phase of other mammalian and bacterial viruses.

Under the experimental conditions used, phase 2 begins with a psychrosensitive step which initiates the replication of Py virus and the induction of cellular DNA synthesis. The experimental data could be explained by a temperature-dependent conformational change of a protein.[24]

Several mammalian viruses are known to undergo functional interactions with the genome of their host cells.[25-27] To our knowledge this is the first analyzed instance of viral infection leading to induction of cellular DNA synthesis. Whether induced host DNA synthesis parallels or is prerequisite to the synthesis of viral progeny is unknown. Py DNA can be considered as a *replicon*,[28] i.e., as a unit of DNA capable of independent replication. As a working hypothesis, we assume that the polyoma-specific initiator might act as an inducer of cellular DNA synthesis in confluent MK cells. Other models are possible and are being experimentally tested.

The molecular events of the early and probably crucial phase of transformation of hamster embryo fibroblasts are not known. It also remains unknown whether infection may release DNA synthesis in some cells continuously from contact inhibition. We assume that the burst of phenotypic (cytogenetic?) variation[3] and the nuclear fragmentation[29] observed shortly after infection are different manifestations of an abortive infection, viral replication being blocked prior to Py DNA synthesis. Loss of cytogenetic equilibrium in transformed cells (references in ref. 29) is regarded as the sequel of the initial events. The presence of new cellular antigens in transformed hamster and mouse cells,[30] as well as the existence of some base sequence homology between DNA from purified Py virus and DNA from transformed though also from normal mouse cells, suggest the possible continued presence of viral DNA in transformed cells.[31] For an interpretation of the results obtained by these homology studies we point to the need to establish the origin of Py DNA III (14S).

The *oncogenic* effect of Py virus shows few, if any, of the characteristics associated with phage conversion in bacteria. It shows similarities, however, to the neoplastic transformation induced by certain chemical carcinogens (references in ref. 29): in both instances loss of cytogenetic equilibrium appears to be one of the crucial events which set the stage for a slow process of variation and selection leading to the appearance of increasingly autonomous cellular variants which are eventually able to escape homeostatic controls.

Summary.—Infection with polyoma virus in confluent mouse kidney cultures proceeds in two distinct phases. During phase 2 the actual replication of progeny virus takes place accompanied by virus-induced host DNA synthesis.

Note added in proof: After submission of this communication, an article by R. Dulbecco, L. H. Hartwell, and M. Vogt came to our attention [these PROCEEDINGS, **53**, 403 (1965)]. These workers also conclude that infection with polyoma virus leads to cellular DNA synthesis in confluent mouse kidney cells.

We are grateful to Professors P. Geiduschek and E. Kellenberger, and to Gret Kellenberger for the discussion of the manuscript. We thank Prof. C. Leuchtenberger and Dr. M. Berchtold for independent microspectrophotometric measurements, Dr. B. Hirt for useful discussions, and Prof. H. Isliker for his interest. The able technical assistance of Mr. P. Wagnière and Miss H. Bursztyn are acknowledged. This work was supported by grants 2366 and 3341 of the Swiss National Foundation for Scientific Research.

[1] Winocour, E., *Virology*, **19**, 158 (1963).
[2] Vogt, M., and R. Dulbecco, *Virology*, **16**, 41 (1962).
[3] Stanners, C. P., J. E. Till, and L. Siminovitch, *Virology*, **21**, 448 (1963).
[4] Defendi, V., submitted for publication.
[5] Leuchtenberger, C., in *General Cytochemical Methods*, ed. J. F. Danielli (New York: Academic Press Inc., 1958), vol. 1, p. 219.
[6] Burton, K., *Biochem. J.*, **62**, 315 (1956).
[7] Weil, R., these PROCEEDINGS, **49**, 480 (1963).
[8] Dulbecco, R., and M. Vogt, these PROCEEDINGS, **50**, 236 (1963).
[9] Weil, R., and J. Vinograd, these PROCEEDINGS, **50**, 730 (1963).
[10] Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis, these PROCEEDINGS, **53**, 1104 (1965).
[11] Watson, J. D., and J. W. Littlefield, *J. Mol. Biol.*, **2**, 161 (1960).
[12] Vinograd, J., R. Bruner, R. Kent, and J. Weigle, these PROCEEDINGS, **49**, 902 (1963).
[13] Vinograd, J., and J. Hearst, *Progr. Chem. Org. Nat. Prods.*, **20**, 372 (1962).
[14] Weil, R., *Virology*, **14**, 46 (1961).
[15] Minowada, J., *Gann*, **55**, 267 (1964).
[16] Vinograd, J., J. Morris, N. Davidson, and W. F. Dove, Jr., these PROCEEDINGS, **49**, 12 (1963).
[17] Setlow, R. B., *J. Cell. Comp. Physiol.*, **64**, 51, Suppl. 1 (1964).
[18] Baldwin, R. L., and E. M. Shooter, *J. Mol. Biol.*, **7**, 511 (1963).
[19] Sheinin, R., *Virology*, **22**, 368 (1964).
[20] Chun, E. H. L., and J. W. Littlefield, *J. Mol. Biol.*, **3**, 668 (1961).
[21] Hakala, M. T., *J. Biol. Chem.*, **234**, 3072 (1959).
[22] Lwoff, A., in *Basic Mechanisms in Animal Virus Biology*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 27 (1962), p. 159.
[23] Gershon, D., and L. Sachs, *Virology*, **24**, 604 (1964).
[24] Monod, J., J. P. Changeux, and F. Jacob, *J. Mol. Biol.*, **6**, 306 (1963).
[25] Franklin, R. M., and D. Baltimore, in *Basic Mechanisms in Animal Virus Biology*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 27 (1962), p. 175.
[26] Joklik, W. K., *J. Mol. Biol.*, **8**, 277 (1964).
[27] Ben-Porat, T., and A. S. Kaplan, *Virology*, **25**, 22 (1965).
[28] Jacob, F., S. Brenner, and F. Cuzin, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 329.
[29] Weil, R., in *Chemotherapy of Cancer, Proceedings of an International Symposium* (Elsevier, 1964), p. 263.
[30] Sjögren, H. O., I. Hellström, and G. Klein, *Cancer Res.*, **21**, 329 (1961).
[31] Axelrod, D., K. Habel, and E. T. Bolton, *Science*, **146**, 1466 (1964).