*ON THE PRODUCTION OF HIGHLY LABELED TUMOR VIRUS DEOXYRIBONUCLEIC ACID**

By Samuel W. Luborsky,† Douglas Lorenz, Dianne Reynolds, John E. Verna, Earl Usdin,‡ and Peter T. Mora†

NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND, AND MELPAR, INCORPORATED, FALLS CHURCH, VIRGINIA

Communicated by Robert J. Huebner, February 16, 1967

This work had as its objective the production of polyoma virus (PV) DNA of high specific radioactivity. Such a highly labeled tumorogenic virus DNA would have many applications in physical, chemical, and biological studies where high sensitivity of detection is essential—for example, for studies on the properties and fate of the viral gene after infection of a cell. This material has been produced[1-4] by adding tritium-labeled thymidine (TdR-H^3) to the growth media of PV-infected cell cultures, since TdR-H^3 label appears only in DNA.[5, 6]

We investigated the effect of 5-fluoro-2′-deoxyuridine (FUdR), an inhibitor of TdR synthesis,[7, 8] in promoting cellular utilization of exogenous high specific activity TdR-H^3. FUdR inhibits DNA synthesis in bacteria[7, 8] and in animal cells.[9-11] Sheinin[11] showed that FUdR inhibited DNA synthesis and PV production in dividing mouse embryo cells. Subsequent substitution of equimolar TdR for the inhibitor reversed this inhibition. No data was presented to show the effect of different levels of FUdR on DNA synthesis in infected cells. Weil[12] observed that addition of FUdR to confluent, PV-infected monkey kidney cell monolayers increased uptake of 5-bromo-2′-deoxyuridine-C^{14} (BUdR-C^{14}), a TdR analogue. Hirt[13] likewise noted that FUdR increased substitution of TdR by BUdR in DNA in PV-infected mouse kidney cultures.

We therefore investigated the effect of different FUdR levels on dividing, uninfected and PV-infected, mouse embryo cells. DNA synthesis was then restored by adding TdR-H^3 to the inhibited system. Virus was purified from the cell cultures, its DNA extracted, and its specific activity determined. We found that labeling of PV DNA was increased by more than threefold in the presence of the drug, to a level nearly sixfold greater than the previously reported.[1]

Materials and Methods.—Cell cultures: Second passage cultures of BALB/c or Swiss albino mouse embryo cells were prepared essentially as described.[14] Hyaluronidase (5 units/ml) (Nutritional Biochemical Corp., Cleveland, Ohio) helped disperse cells during the first and/or the second trypsinizations, for preparation of primary cultures.[15] About 2–3 $\times$ 10^6 cells in Eagle's medium[16] with 10% serum (horse or fetal calf) were seeded onto 100-mm plastic Petri dishes. Some cultures, suitably scaled-up, were grown in 8- or in 32-oz prescription bottles. Confluent monolayers were observed at about 24–30 hr. No differences were noted due to mouse strain or type of serum used in various experiments. FUdR and TdR were incorporated in the growth medium added after PV infection.

Virus: A wild-type strain of PV was obtained through the courtesy of Dr. Robert Ting. This strain originated in Rowe's laboratory and was plaque-purified in Dulbecco's laboratory. Various preparations were grown in mouse kidney or in mouse embryo cells.[17] Virus was adsorbed for 2 hr at a multiplicity of 10–30 PFU/cell, about 30 hr after seeding. Virus was assayed by plaque formation.[14]

Labeling with TdR-H^3: High specific activity TdR-H^3: 0.2 ml (160 μc) of the commercially available TdR-H^3 stock (1.6 $\times$ 10^4 μc/μmole) added to 10 ml of growth medium, produced a 10^{-6} *M* solution. Labeled cytidine (CdR-H^3) (603 μc/μmole) was also added to the media at

10^{-6} *M*. Lower activity TdR-H^3: 1 ml of a diluted stock solution added to 10 ml of media. This diluted stock solution contained 10^{-2} *M* TdR and 10^{-7} *M* TdR-H^3.

Tritium incorporation into total DNA was determined after precipitation of a frozen-thawed cell suspension in 5% perchloric acid. The cold methanol-washed precipitate was dissolved in 0.2 ml CSC solubilizer (Nuclear-Chicago Corp., Chicago, Ill.) and added to 10 ml of a toluene scintillation mixture. Radioactivity in column chromatographic fractions was measured in Bray's solution.[18] Virus purification and PV DNA extraction were monitored by counting aliquots of the solutions on Whatman No. 1 paper in the toluene scintillation mixture. Count rates were determined in a Packard Tri-Carb liquid scintillation spectrometer, at a counting efficiency for the H^3-toluene standard of 22%.

Isotopically labeled compounds were obtained from New England Nuclear Corp., Boston, Mass.

Chromatography of DNA: Methylated albumin-coated kieselguhr (MAK) columns were used.[19] Samples were applied in 0.1 *M* NaCl, and eluted in a linear salt gradient (0.1–1.0 *M* NaCl). Salt concentrations were determined from the conductivity of the fractions.

Virus purification: This procedure was modified from Winocour.[17] At 4–4$^1/_2$ days after infection, growth medium was discarded, cells were trypsinized, frozen-thawed, and virus-containing cell debris collected by centrifugation. After sonication in a small volume of supernatant solution (S), the pellet was incubated[20] with 1 unit/ml neuraminidase (Calbiochem, Los Angeles, Calif.). Meanwhile, to reduce volume, the remaining virus was sedimented from aliquots of S, dispersed in a small volume of S, and added to the incubation mixture for the last 1–2 hr. Finally, large aggregates in this mixture were dispersed using a Teflon homogenizer, the debris sedimented, and the clarified suspension mixed with any remaining S. Sodium pyrophosphate was added (to 0.1 *M*) and the solution incubated with 10 μg/ml RNase (30 min, 37°C) to degrade ribosomal particles,[21] before sedimenting the virus. The pellet, resuspended in 2 ml of 0.01% trypsin, was incubated briefly, 0.904 gm CsCl added, and this solution, mixed with 6 ml of 2.68 molal CsCl, was centrifuged to equilibrium. Fractions (1.30–1.33 gm/ml) were combined, diluted with buffer, and the virus sedimented. The pellet was resuspended in 0.01% trypsin, DNase, and $MgCl_2$ added (to 10 μg/ml, and 0.003 *M*, respectively), and the volume brought to 1 ml. After incubation, 0.452 gm CsCl was added, the solution diluted to 4 ml with 2.68 molal CsCl, and again centrifuged to equilibrium. Appropriate fractions were combined and dialyzed overnight against three changes of Weil's[22] buffer (1.5 ml of versenate buffer, 2.5 ml of potassium trichloroacetate solution) before phenol extraction.

DNA extraction: DNA was extracted from this purified virus by two successive phenol extractions. The phenol phases were each twice back-extracted with Weil's buffer.[22] Combined aqueous phases were dialyzed 22 hr against five changes of 0.1 × SSC (0.1 × 0.15 *M* NaCl, 0.015 *M* sodium citrate). Volume was reduced sevenfold by covering the dialysis bag with washed, dry Sephadex G-75 before opening.

DNA was extracted from cells by a modified pronase, sodium lauryl sulfate (SLS), phenol procedure.[23] The use of pronase increased our recovery of DNA. Trypsinized, frozen-thawed cells were suspended in SSC to which 2 mg/ml pronase (Calbiochem, Los Angeles, Calif.) and 1 mg/ml SLS were added. After incubation (30°C, 18–22 hr) and phenol extraction (5°C), combined aqueous phases were dialyzed against 0.1 *M* NaCl, 0.05 *M* sodium phosphate, pH 7.

DNA preparations were stored at −70°C.

DNA estimation: DNA was estimated by Giles and Meyers'[24] modification of Burton's diphenylamine reaction,[25] scaled down 20-fold by lyophilizing the DNA preparation to dryness and dissolving the residue in 0.1 ml 10% perchloric acid (PCA). Absorbancies were read using microcuvettes. Calibration curves obtained in this fashion, using calf thymus DNA as a standard, were routinely reproducible down to 0.5 μg per tube.

Results.—Appropriate conditions were sought for the addition of FUdR and TdR-H^3 to our cultures. Infection with PV (−FUdR) lead to a stimulation of almost 1.5-fold in the rate of DNA synthesis (Fig. 1*a*). In the presence of FUdR, incorporation was inhibited to comparable levels in both infected and uninfected cultures. We chose 10^{-4} *M* FUdR as a concentration high enough to produce an appreciable inhibition of DNA synthesis (*ca.* 90%) yet not sufficient to reduce the cell count. Moderate amounts of TdR (Fig. 1*b*) stimulated DNA synthesis in the

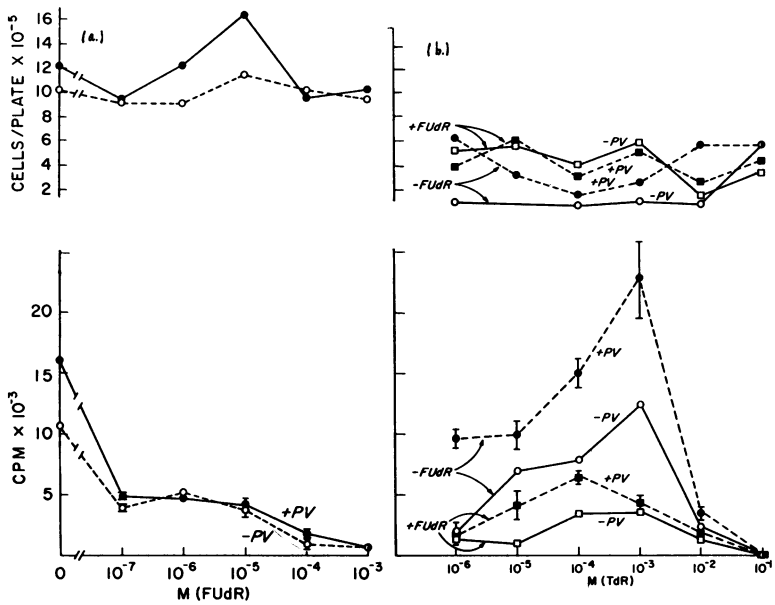


FIG. 1.—Effect of FUdR and TdR on DNA synthesis in infected (+PV) and uninfected (−PV) mouse embryo cultures. Synthesis was measured by incorporation of H^3 into total acid-precipitable material per culture following a CdR-H^3 pulse, 22–24 hr after infection. Each point represents an average of measurements on duplicate plates (extremes represented by bars). Cells were counted in a hemocytometer. (*a*) Inhibition of incorporation by FUdR; (*b*) restoration of incorporation by TdR, in the presence of 10^{-4} *M* FUdR.

presence of FUdR. High TdR levels completely inhibited DNA synthesis, whether or not the cells were infected. Inhibition by high concentrations of TdR has been reported.[26, 27] Efficient restoration of DNA synthesis occurred at 10^{-3} *M* TdR in both infected and uninfected cultures.

The time course of DNA synthesis in the presence of these levels of FUdR and TdR, with a small amount of TdR-H^3 added to label the DNA synthesized, is shown in Figure 2. Note the 24 hour time, chosen to determine the rate of DNA synthesis (Fig. 1), is the time at which incorporation reaches its maximum. The decrease after 96 hours is due principally to loss of cells from the cultures. FUdR markedly inhibits total incorporation; PV infection has little effect. A suitable time for harvesting virus is 96 hours after infection, while incorporation levels are still maximal and no cytopathic effect (CPE) of virus on the cells has appeared. Since most of the virus is within the cells at times prior to extensive CPE,[14] such an early harvest time permits one to concentrate the cells before rupturing (e.g., ref. 6), and thus obtain a more concentrated virus suspension. Moreover, the use of infected cell monolayers alone eliminates serum-containing growth medium. This medium interferes with subsequent phenol extraction when using such ions as trichloroacetate.[22]

MAK column chromatography[19] was employed to separate viral from cellular DNA in extracts of infected cells in an attempt to characterize the DNA produced. The large columns used gave nearly complete separation of a synthetic mixture (Fig. 3), at the expense of lower recoveries of input radioactivity than reported by other workers.[5, 28, 29] Admixture of cellular DNA affected neither the recovery nor

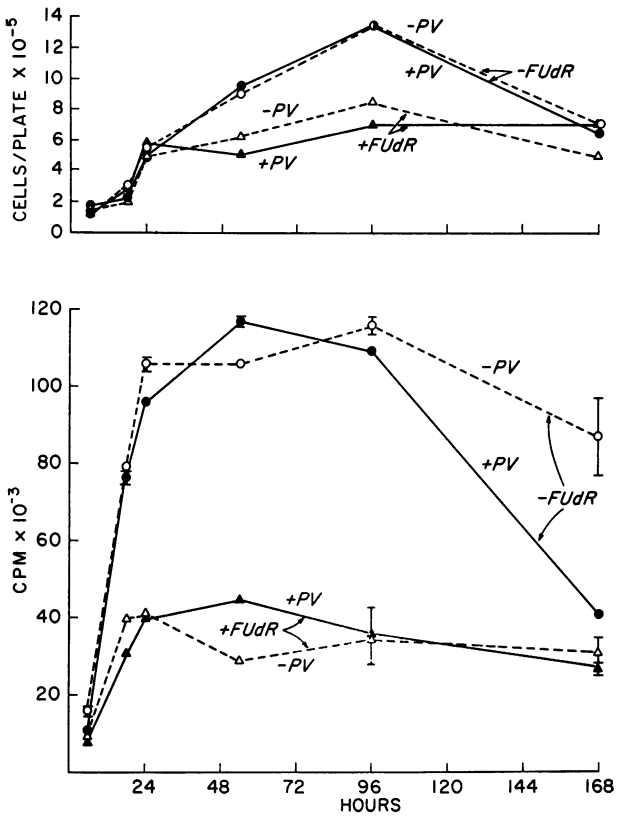


FIG. 2.—Time course of incorporation of TdR-H³ into mouse embryo cultures in the presence of 10^{-4} *M* FUdR, 10^{-3} *M* TdR, and 10^{-6} *M* TdR-H³ (1.6×10^4 μc/μmole), added to cells at time of infection (zero hr), and present continuously thereafter. Precipitation and cell count performed as noted in legend to Fig. 1.

the molarity of salt at which viral DNA was eluted from the column. For various preparations and column runs PV DNA eluted at 0.4–0.6 *M* NaCl; cellular DNA, at 0.6–0.8 *M*.

Figure 4 is a composite of results, obtained in different runs with DNA extracted from infected cells grown in diluted label, superimposed to show the effect of FUdR upon growth. The peaks superimpose quite well. FUdR decreases uptake of label. The amount of DNA present at each time agrees with that predicted (Fig. 2). In all cases, most radioactivity elutes at 0.7–0.8 *M* salt, where cell DNA would be expected to appear. At 36 hours (+FUdR), and at 96 hours (−FUdR), extra peaks appear at 0.5–0.6 *M* NaCl, where PV DNA would be expected to appear.

DNA extracted from PV-infected cells labeled with 10^{-6} *M* high specific activity TdR-H³ alone (1.6×10^4 μc/μmole) (+FUdR), gave the elution profiles shown in Figure 5. The specific activity of this DNA extract was 67,000 cpm/μg DNA, compared to 1,100 cpm/μg obtained using diluted label (Fig. 4). Elution is predominantly at the position expected for PV DNA, whereas no such peak appeared at the 24 hour time after infection in diluted TdR-H³.

Finally, production of highly labeled, purified PV DNA was carried out under the conditions chosen. Note the high specific activity obtained (Table 1), especially in the presence of FUdR.

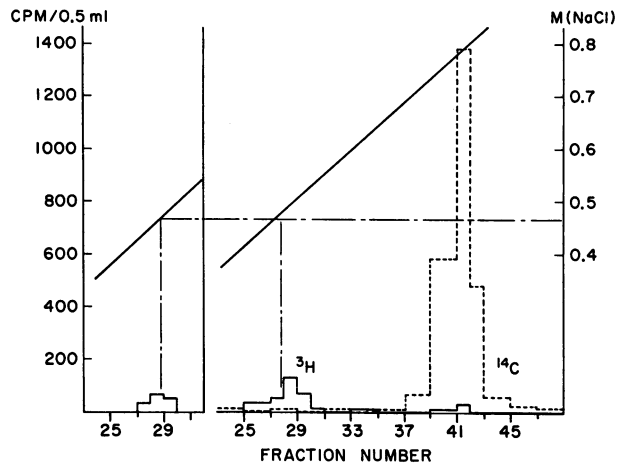
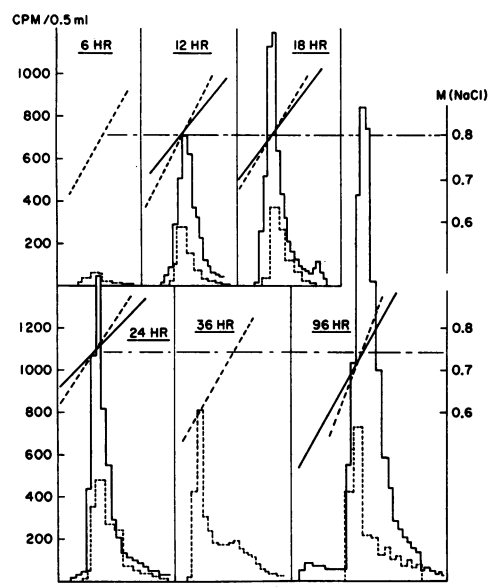
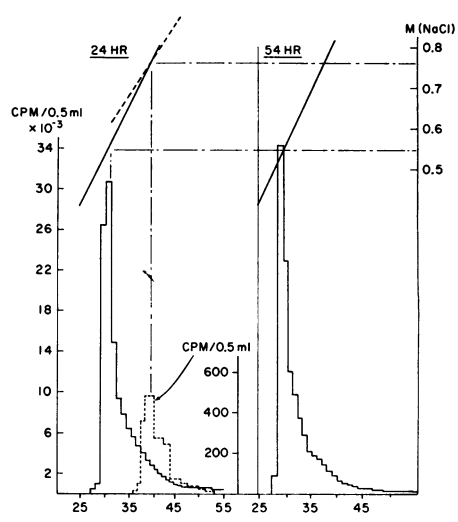


FIG. 3.—MAK column chromatography of PV DNA-H^3 and of cellular DNA-C^{14} from mouse embryo cultures. PV-DNA was obtained by phenol extraction of a PV preparation grown with TdR-H^3 and purified by CsCl isopycnic centrifugation. Cellular DNA was phenol-extracted from uninfected cultures grown with TdR-C^{14}. Column size 2.0 × 12.5 cm; flow rate 25 ml/hr; fraction volume, 5 ml. *Left:* PV DNA-H^3: input, 12,500 cpm; recovery, 2,500 cpm. *Right:* Same PV DNA-H^3: mixed with cellular DNA-C^{14}: input, 20,000 cpm H^3, 40,000 cpm C^{14}; recovery, 4000 cpm H^3, 12,000 cpm C^{14}.





(*Left*) FIG. 4.—MAK column elution profiles of DNA-H^3 obtained from PV-infected cells at various times after infection. Growth in 10^{-4} *M* FUdR, - - -; growth without FUdR, ——. Column conditions, cf. Fig. 3. Patterns superimposed from separate column runs so that salt concentration at which one peak elutes (−FUdR) coincides with same salt concentration in the other pattern (+FUdR). To simplify representation, fraction numbers have been omitted, since interest lies in the molarity of salt at which a peak elutes. Growth, infection, and labeling conditions, cf. Fig. 2. The 36-hr (+FUdR) measurement was omitted from the original experimental protocol. Three infected cultures were pooled for each time point; 1/3 of extracted DNA was placed on column. Recoveries for various columns were in the range 35–60% of input radioactivity.

(*Right*) FIG. 5.—MAK column elution patterns for PV-infected cell DNA at two times after infection. Growth in 10^{-4} *M* FUdR and 10^{-6} *M* TdR-H^3 (1.6×10^4 $\mu c/\mu$mole). - - -, superimposed from Fig. 4, for comparison.) All other conditions, cf. legend, Fig. 4. Recovery, 50% of input.

Discussion.—We have obtained highly radioactive H^3-DNA from PV by growing virus in the presence of FUdR (10^{-4} *M*) and 1.6×10^4 $\mu c/\mu$mole TdR-H^3 (10^{-6} *M*). Although 50 per cent less PV DNA is produced in the presence of this amount of

TABLE 1

PRODUCTION OF HIGHLY LABELED PV DNA

	DNA (μg)	Total cpm	Cpm/μg
+FUdR	15 ± 1	2,670,000	178,000
−FUdR	36 ± 2	2,050,000	57,000

Two groups of 100 PV-infected cultures each, derived from BALB/c mice, grown at 10^{-6} *M* TdR-H^3 (1.6×10^4 μc/μmole), (*a*) in the presence (10^{-4} *M*), or (*b*) in the absence, of FUdR. Solutions harvested 4½ days later had infectivities of 2–5 × 10^7 PFU/ml, after freezing and thawing. Specific activity was determined as described in text. Incorporation was measured in other PV preparations grown in cells derived from Swiss albino, as well as from BALB/c, mice. Some of these were less well purified or from smaller pools, yielding too little DNA to measure with confidence. Nevertheless, qualitatively similar results were obtained in each case.

FUdR and TdR-H^3 (Table 1), it is over three times more highly labeled than in the absence of the drug—a level nearly six times greater than that previously reported.[1] In keeping with our original hypothesis, use of FUdR has enhanced incorporation of TdR-H^3 into PV DNA.[30]

It is unlikely that spurious radioactivity is responsible for this result. Both preparations were treated in an identical fashion, and the −FUdR preparation yielded a specific activity not much higher than that already reported.[1] It is possible that FUdR increased the amount of labeled cell debris contaminating the +FUdR virus solutions. Notwithstanding, the purification procedure used should have adequately removed such contaminants, based as it was upon methods investigated and used by other workers,[6, 17, 20] and including treatments with pyrophosphate and RNase,[21, 28] DNase, and trypsin,[31] and two cycles of isopycnic centrifugation. Moreover, cellular DNA contaminating the virus would be of lower specific activity than PV DNA and could not fortuitously increase the specific activity of the DNA extract.

FUdR has not previously been used under comparable conditions. Sheinin[11] used 10^{-5} *M* FUdR to reduce DNA synthesis in mouse embryo cultures to about 5 per cent at 24 hours. Under our conditions, it was necessary to use 10^{-4} *M* FUdR to attain nearly the same level of inhibition. Hirt[13] utilized 6×10^{-5} *M* FUdR to inhibit thymidylate synthesis in more than 99 per cent of PV-infected mouse kidney cells.[32]

Our FUdR level was chosen on the basis of experiments in which it produced inhibition of total DNA synthesis without grossly affecting cell viability at 24 hours—as measured roughly by the similarity of cell counts in treated and untreated cultures at this time. That a more appropriate criterion might be used is evident from the reduced viability subsequently observed. Although their growth rate was the same as the control up to 24 hours (Figs. 1 and 2), analogue-treated cultures incorporated less label at 24–96 hours. Perhaps this FUdR toxicity[33] is responsible for the reduced yield of PV DNA (Table 1), and for the slight continuing irreversible inhibition of DNA synthesis evident when TdR is added to the cultures (Fig. 1*b*). These effects are somewhat irrelevant, however, since our original objective, a high level of incorporation into viral DNA, was achieved.

Incorporation of TdR-H^3 into DNA of mammalian cells can destroy their capacity for continuous proliferation.[35] It is difficult to assess the effect of this inhibition on virus yield or extent of TdR-H^3 incorporation into PV. This reduction in cell viability may be related to the radiation-induced lability introduced into DNA chains by H^3-incorporation, presumably[36] through introduction of single-strand

chain scissions in the DNA. Certainly this type of lability would limit the usefulness of any highly labeled virus preparations where molecular integrity of the DNA chain is of importance.

Most TdR-H^3 incorporation should be into cellular DNA, since we were working at a low multiplicity of infection. Moreover, there is a stimulation of cellular DNA synthesis following PV infection.[5, 12, 37] Our MAK column data (Figs. 4 and 5) confirms this expectation, and also the general conclusions drawn from the time-course experiments (Fig. 2).

Although we might expect to see the same kind of breakdown in most profiles, the small peaks at the position expected for viral DNA (Fig. 4) could be due to either fortuitous or radiation-induced breakdown[36] of cellular DNA. Furthermore, in spite of its elution position the highly labeled DNA (Fig. 5), could be predominantly degraded cellular DNA. This radioactive DNA, having a much higher level of H^3-incorporation, might be expected to be much more labile than the former.[38, 39] These results underscore the pitfalls inherent in interpretation of data obtained using procedures on highly labeled DNA that depend upon differentiation in molecular size.

The methods used in this study should be applicable to the production of other highly labeled DNA viruses in tissue culture.

Summary.—A method is described for obtaining highly radioactive PV DNA. FUdR at 10^{-4} *M* together with TdR-H^3 (1.6×10^4 μc/μmole) at 10^{-6} *M* were used in the growth media of PV-infected mouse embryo cell cultures. After extensive purification of the resulting virus, its extracted DNA exhibited a specific activity of 178,000 cpm/μg, over three times higher than that observed for PV grown under similar conditions in the absence of FUdR.

We thank Drs. Robert Ting for supplying virus seed, W. E. Scott of Hoffman-LaRoche for donating FUdR, T. O'Connor for advice on plaque assay procedure, John Bader for many fruitful discussions, and Drs. Paul Black, Bill H. Hoyer, and Sherman Weissman for comments on the manuscript. Dr. Roger Wood was kind enough to purify some virus samples for us.

* Part of this work, performed at Melpar, Inc., was supported by NIH contract PH43-64-1162.

† S. W. L. and P. T. M. at National Cancer Institute.

‡ Present address: Atlantic Research Corporation, Alexandria, Virginia.

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[30] Maximum specific activity for PV DNA can be estimated as about $3–5 \times 10^6$ cpm/μg, assuming complete equilibration of (1.6×10^4 μc/μmole) TdR-H^3 between growth medium and cells. Our observed value, 3–5% of this theoretical maximum (Table 1) (+FUdR), is consistent with labeling of the DNA in about $1:10^6$ virions.

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[33] Continuing FUdR toxicity has been noted, for example, in both HeLa[9] and L cell[34] systems, pretreated for 16 hr with FUdR. Subsequent addition of 10^{-5} *M* TdR reversed the effect of 10^{-6} *M*, but not that of 10^{-5} *M*, FUdR. Since some FUdR is converted to fluorouracil and its derivatives, which inhibit RNA metabolism, it was suggested[9] that such side reactions might contribute to the toxicity of FUdR at elevated levels. Since our FUdR level was even higher, this explanation might apply to our system, even though both FUdR and TdR were added simultaneously.

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[38] More work is required to characterize this DNA. Band sedimentation through CsCl solutions of density 1.50 gm/ml yielded inconclusive results. There had been a lapse of about 3 weeks between MAK column and band sedimentation experiments, during which H^3-decay could have produced further degradation of DNA chains.[36] Tentative support for this suggestion was provided by the shift in sedimentation patterns observed for duplicate samples run at the beginning and the end of this period.

[39] The behavior of the latter DNA might also reflect the unstable "cellular" DNA reported[40] in PV-infected confluent cultures, which is degraded to the approximmate size of PV DNA.

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