

Preparation of Poliovirus Labeled with Phosphorus-33

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Phosphorus-33 (³³P), a weak (0.25 Mev) beta-emitting isotope of phosphorus with a half-life of 25 days, has been used to label poliovirus in cell culture. HeLa cell monolayers were depleted of phosphate and then labeled by incubating at 37 C in a medium (LM) containing about 10 μ Ci of ³³P as orthophosphate per ml. Labeled cells were infected at a high multiplicity with poliovirus type 1 and incubated for 8 hr in LM medium. Virus from infected cells was then concentrated and purified. Virus purity was confirmed by comparison of virus infectivity and radioactivity after CsCl density gradient centrifugation and by observing purified virus preparations with electron microscopy. With the method described, yields of about 10¹⁰ to 5 \times 10¹⁰ plaque-forming units (PFU) of highly purified poliovirus with specific activities of about 3 \times 10⁻⁴ to 10⁻³ disintegrations per min per PFU have been obtained from 1.5 \times 10⁸ to 3.0 \times 10⁸ HeLa cells.

The labeling of animal viruses with radioisotopes is now a widely used technique with a variety of applications (3, 10). The only isotopes used extensively, namely, ³H, ¹⁴C, ³²P, and ³⁵S, are those whose corresponding stable isotopes are important components of viral and other biological macromolecules.

Although ³²P has frequently been used to label animal viruses in their nucleic acid (4, 5, 11), ³³P, which is now available from commercial sources in acceptable radioisotope purity, appears to offer several advantages over ³²P as a radiotracer for phosphorus in viruses and other biological materials (8). Both ³²P and ³³P decay by beta emission to produce stable sulfur atoms. The half-life of ³³P is longer than that of ³²P, 25 versus 14.3 days, permitting longer term experiments and lengthening the usability of labeled samples. Phosphorus-33 has a lower maximum energy of beta emission than ³²P, 0.25 versus 1.71 Mev, resulting in a lower recoil energy and lessening the external dose to the user. In addition, ³³P permits double phosphorus labeling experiments by using conventional techniques such as liquid scintillation counting.

To evaluate ³³P as a radiotracer for phosphorus in animal viruses, a series of studies on the preparation and characterization of ³³P-

labeled animal viruses was conducted. This paper describes a simplified method for the preparation of poliovirus labeled with ³³P and some characteristics of the labeled virus.

MATERIALS AND METHODS

Cell culture. HeLa cells were grown as monolayers in autoclavable minimum essential medium (AMEM; Flow Laboratories, Inc., Inglewood, Calif.) containing 10% heat-inactivated fetal calf serum (FCS), 100 units of penicillin per ml, and 100 μ g of streptomycin per ml. Cells from confluent monolayers in 32-oz (ca. 0.95 liter) prescription bottles were dispersed with 10- to 20-ml volumes of 0.02% ethylenediaminetetraacetic acid (EDTA) in calcium- and magnesium-free phosphate-buffered saline (9). The dispersed cells were centrifuged at 1,000 \times g for 10 min, the supernatant fluid was discarded, and the sedimented cells were suspended in growth medium at a concentration of about 5 \times 10⁵ cells/ml. Cell cultures for virus propagation were prepared by inoculating 50-ml volumes of cell suspension into 32-oz prescription bottles, and cell cultures for plaque assay were prepared by inoculating 4-ml volumes of cell suspension into 1-oz (ca. 0.03 liter) prescription bottles. Cell cultures were incubated at 37 C. Confluent monolayers, which formed in 4 to 6 days, were maintained in AMEM with 3% FCS, 100 units of penicillin per ml, and 100 μ g of streptomycin per ml.

Virus. Poliovirus type 1 strain LSc was used exclusively in this study. The virus was plaque-purified and passaged several times in HeLa cells. For passage, drained HeLa-cell monolayers were infected with virus at a multiplicity of about 20. After a 30-

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TABLE 1. *Some characteristics of purified, ³²P-labeled poliovirus preparations*

Prepn no.	Total ³² P (mCi)	³² P concn in labeling medium (μCi/ml)	No. of HeLa cells	Total virus infectivity (PFU) ^a	Total virus radioactivity (dpm) ^b	Specific activity (dpm/PFU)	No. of ³² P atoms/PFU
1	5.0	16	3.0 × 10 ⁸	9.1 × 10 ⁹	1.2 × 10 ⁷	1.3 × 10 ⁻³	68
2	2.0	12	1.5 × 10 ⁸	1.2 × 10 ¹⁰	9.1 × 10 ⁶	7.6 × 10 ⁻⁴	40
3	3.0	8	3.0 × 10 ⁸	1.2 × 10 ¹⁰	1.1 × 10 ⁷	9.2 × 10 ⁻⁴	48
4	3.0	10	2.7 × 10 ⁸	4.6 × 10 ¹⁰	1.3 × 10 ⁷	2.8 × 10 ⁻⁴	15

^a Plaque-forming units.^b Disintegrations per minute.TABLE 2. *Properties of ³²P-labeled poliovirus during stages of purification*

Sample	Total infectivity (PFU) ^a	Total radioactivity (dpm) ^b	Specific activity (dpm/PFU)
Labeling medium		1.1 × 10 ¹⁰	
Cell lysate after cell debris removed	6.2 × 10 ¹⁰	3.0 × 10 ⁹	4.8 × 10 ⁻²
Resuspended virus pellet	1.0 × 10 ¹⁰	2.3 × 10 ⁷	2.3 × 10 ⁻³
Purified virus	9.1 × 10 ⁹	1.2 × 10 ⁷	1.3 × 10 ⁻³

^a Plaque-forming units.^b Disintegrations per minute.

min adsorption period at 37 C, the cell sheets were washed twice with 20-ml volumes of maintenance medium made with gamma globulin-free calf serum (GFCS). Then, 30 ml of maintenance medium made with GFCS was added to each bottle, and infection was allowed to proceed for 8 hr at 37 C. The medium was removed, and the virus-infected cells were washed and collected in a small volume of 0.02 M phosphate buffer, pH 7.1. This crude virus-cell harvest was subjected to 5 cycles of freezing and thawing, centrifuged at 10,000 × *g* for 10 min in the cold to remove cell debris, and stored at -70 C until used.

Preparation of ³²P-labeled poliovirus. The procedure for labeling poliovirus with ³²P was similar to previously described methods for labeling poliovirus with ³²P (4, 5) and involved the following steps. Confluent HeLa cell monolayers in 32-oz bottles were depleted of phosphate by washing twice with phosphate-free medium (PFM) and then incubating for 5 hr at 37 C with hourly medium changes. PFM consisted of citrate-buffered, phosphate-free minimum essential medium containing 3% GFCS which had been dialyzed to remove phosphate. After phosphate depletion, the cells were relabeled with ³²P by replacing PFM with a labeling medium (LM), consisting of PFM with 10 μCi of ³²P as orthophosphate per ml, and incubating for 5 hr at 37 C. After prelabeling, the medium was removed, and the cells were infected at a multiplicity of 10 to 30 with poliovirus which had been dialyzed against PFM. Virus was allowed to adsorb for 30 min at 37 C, and the mono-

layers were washed twice with PFM to remove unadsorbed virus. LM was added to the cells, and infection was allowed to proceed for 8 hr at 37 C. The medium was then removed, and the virus-infected cells were washed and collected in a small volume of 0.02 M phosphate buffer, pH 7.1. This crude, ³²P-labeled virus harvest was purified by the method of Phillips, Summers, and Maizel (7). Briefly, the purification procedure involved 5 cycles of freezing and thawing, treatment with 1% sodium dodecyl sulfate, centrifugation at 10,000 × *g* to remove cell debris, centrifugation at 120,000 × *g* to pellet the virus, and banding of the virus in a CsCl (1.3 g/cc) density gradient. Virus was collected by puncturing the bottom of the centrifuge tube and collecting 5-drop fractions in 3 ml of 0.02 M phosphate buffer.

Confirmation of virus purity. The purity of ³²P-labeled virus was confirmed by comparison of infec-

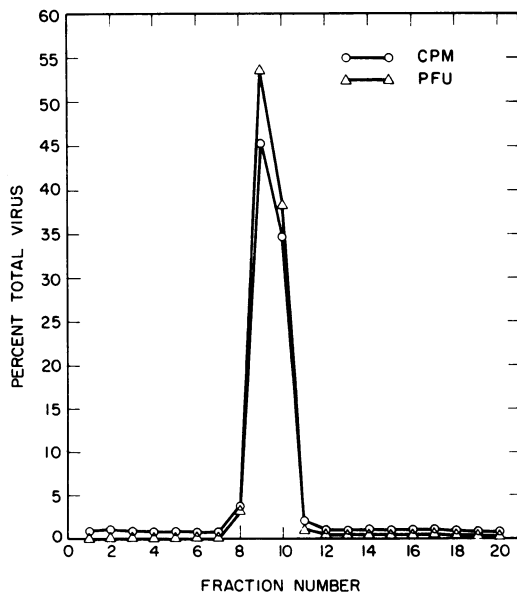


FIG. 1. *Distribution of infectivity and radioactivity after CsCl density gradient centrifugation of ³²P-labeled poliovirus.*

tivity and radioactivity after equilibrium centrifugation in a CsCl density gradient and by observing purified virus preparations with electron microscopy. A 4.5-ml sample of purified, labeled virus in 0.02 M phosphate buffer, pH 7.1, containing 1.3 g of CsCl per cc, was placed in a 5-ml capacity cellulose nitrate tube and centrifuged at $120,000 \times g$ for 16 to 18 hr at 4 C. Virus was harvested from the bottom of the tube in 5-drop fractions and assayed for virus infectivity and radioactivity. For electron microscopy, a sample of purified virus was placed on a collodion-filmed grid, shadow cast with uranium, and examined with a Siemens Elmiskop 1A electron microscope.

Virus infectivity assay. Poliovirus was assayed by the plaque technique on HeLa cell monolayers. Samples from experiments were diluted serially 10-fold in maintenance medium made with GFCS. Each of three drained cell sheets was inoculated with 0.2 ml of a sample dilution, and virus was allowed to adsorb for 45 min at 37 C. During the adsorption period, the inoculum was respread on the cell sheets every 15 min by rocking the bottles. The bottles were then inoculated with 4 ml of an overlay medium consisting of 1.8% agar (Difco), 3% GFCS, 0.2% NaHCO₃, and 25 mM MgCl₂ in AMEM without phenol red. After 2 days of incubation at 37 C, the developed plaques were stained for counting by adding 1 ml of 0.08% neutral red to each bottle and incubating for 4 hr at 37 C. The concentration of virus was expressed as plaque-forming units (PFU)/ml.

Radioactivity assay. One-half milliliter of a radioactive sample was added to 10 ml of Brays solution (2) and counted in a Nuclear Chicago unilux II liquid scintillation spectrometer. Radioactivity measurements were expressed as counts per minute (cpm) and corrected for background. Instrument efficiency was determined with a ³²P standard source to estimate the absolute activity of samples in terms of disintegrations per minute (dpm). Carrier-free ³²P as orthophosphate was obtained from Tracerlab, Waltham, Mass.

RESULTS

Some characteristics of four different preparations of ³²P-labeled poliovirus are shown in Table 1. The concentration of ³²P in the LM ranged from 8 to 16 μ Ci/ml, and the number of HeLa cells used ranged from 1.5×10^8 to 3.0×10^8 cells. Under the experimental conditions used, virus yields of 9.1×10^9 to 4.6×10^{10} PFU were obtained, and the specific activities of the preparations ranged from 1.3×10^{-3} to 2.8×10^{-4} dpm/PFU. The number of ³²P atoms per PFU ranged from 15 to 68.

Table 2 shows the infectivity, radioactivity, and specific activity of virus preparation 1 at various stages of purification. As nonviral ³²P was removed with each successive stage of pu-

rification, the amount of radioactivity in the labeled virus preparation decreased. About 15% of the initial amount of crude virus was recovered after purification, and about 0.1% of the original ³²P was associated with purified virus. In virus preparations 2, 3 and 4, the percent of initial virus recovered after purification was 36, 32 and 70%, respectively. The low recovery in preparation 1 may have been due to poor technique in this initial experiment.

The labeled virus of preparation 1 was checked for purity by banding in a CsCl density gradient. As shown in Fig. 1, most of the virus infectivity and radioactivity was associated with the virus band located in the center of the tube, and there was a good correspondence between the percentages of infectivity and radioactivity present in each fraction.

Figure 2 shows an electron micrograph of poliovirus purified by the previously mentioned procedure. Since little cell debris or other nonviral material was observed, electron microscopy provided additional confirmation of virus purity.

DISCUSSION

The results of this investigation indicate the poliovirus can be radioisotopically labeled with ³²P by methods similar to those used for labeling viruses with ³²P. This finding was not unexpected since several investigators have labeled bacteria and bacteriophages with ³²P (1, 6). By using a recently reported technique, the labeled virus can be rapidly and easily purified. It should be possible to label other animal virus with ³²P. It might also be expected that for certain ribonucleic acid viruses the specific activities of ³²P-labeled virus preparations could be increased by using an inhibitor of cellular ribonucleic acid synthesis such as actinomycin D. This has been observed when ³²P is used as the virus label. Phosphorus-33 may prove to be a useful radioisotopic tool for virus research.

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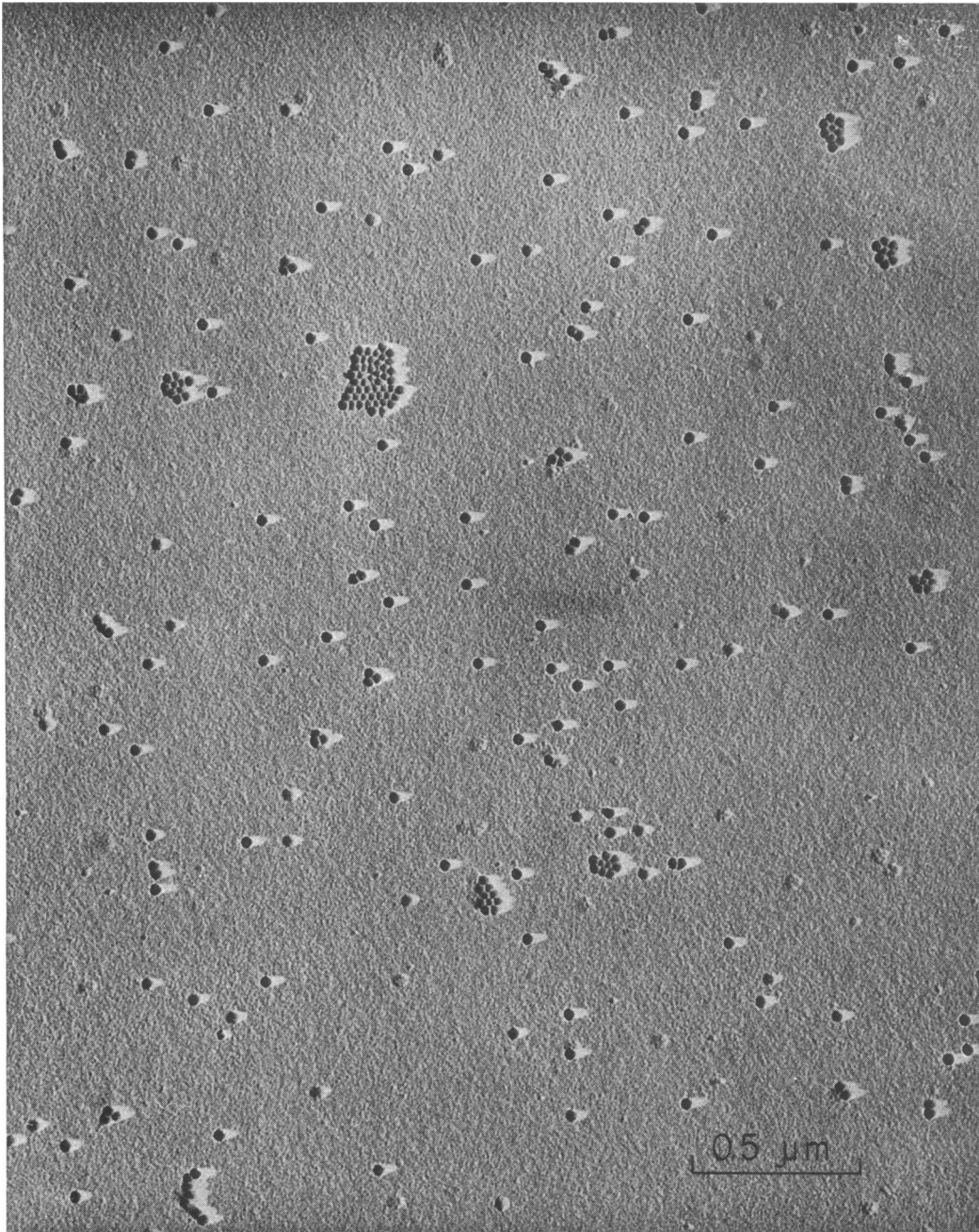


FIG. 2. *Electron micrograph of poliovirus type 1 purified by the method described in Materials and Methods* $\times 20,000$.

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