Taste (ed., Y. Zotterman), Pergamon Press, New York, 1963, 285.

6. Richter, C. P., Campbell, K., J. Nutr., 1940, v20, 31.

7. Kare, M. R., in: Physiological and Behavioral Aspects of Taste (M. R. Kare & B. P. Halpern, ed.), Univ. of Chicago Press, Chicago, 1961, 6.

8. Benedict, F. B., Petrick, J. M., Am. J. Physiol., 1930, v94, 662.

9. Booth, A. N., Wilson, R. H., Deeds, F., J. Nutr., 1953, v49, 347.

10. Yudkin, A. M., Geer, H. A., Arch. Ophthalmol., 1940, v23, 28.

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Virus-Free Infective Centers Produced by Photodynamic Inactivation of Poliovirus in Rhesus Kidney Cell Suspensions. (30136)

C. W. HIATT AND DOROTHY E. MOORE*

Division of Biologics Standards, National Institutes of Health, Bethesda, Md.

Poliovirus propagated in cell cultures containing proflavine, acridine orange, or neutral red becomes sensitive to light(1-3) and retains this sensitivity as a phenotypic tag (4), which may be used to distinguish between successive generations of virus. Several investigators(3,5,6,7) have employed photosensitive poliovirus as a means of studying the early course of infection of the cell, making use of the fact that irradiating the system with visible light will destroy parent virus without affecting the cells or any virus progeny which may have been present at time of irradiation.

Wilson and Cooper(3,7) found that poliovirus sensitized with neutral red or acridine orange loses its photosensitivity soon after adsorption to susceptible cells and that this loss is not due simply to entry into the cell, but results from something which happens to the virus particle in a later stage of the process of infection. McBride's earlier observations(5) as well as those reported concurrently by Schaffer and Hackett(6) differ somewhat in experimental detail but lead essentially to the same conclusion.

In similar experiments of our own, we have found that, by irradiating cell suspensions promptly after adsorption of proflavine-sensitized poliovirus, we can obtain cellular centers of viral infection from cell suspensions which contain no immediately recoverable virus. This demonstration of a total eclipse phase of poliovirus infection is of interest because of its implications about the transfer of genetic information from the virus to the synthetic apparatus of the cell.

Methods. Preparation of virus. Poliovirus, type 1, Mahoney, was sensitized to light by 3 serial passages in the dark in monolayers of primary rhesus kidney cells in medium 199 (8) containing 2% calf serum and 2 mcg proflavine per ml. Plaque-forming units (PFU) were determined on confluent monolayers of primary rhesus kidney cells.

Preparation of cells. Monolayer cultures of Hull's $LLCMK_2$ stable rhesus kidney cell line(9) were prepared in 32-oz bottles. After a preliminary washing with 10 ml of Hanks' balanced salt solution (HBSS)(10), the cell sheet from each bottle was dislodged from the glass by incubation for 15 minutes at 36°C with 20 ml of 0.15 M NaCl containing 0.25% trypsin. The yield from 4 bottles was pooled, sedimented by centrifugation for 5 minutes at 1500 rpm and resuspended in 5 ml of HBSS containing 0.5% lactalbumin. The cell concentration was determined using a Coulter counter or by visual counting in a hemocytometer using trypan blue stain.

Experimental procedure. An inoculum of 0.4 ml of photosensitized poliovirus containing 10^7 plaque-forming units (PFU) was added to a suspension of 10^7 cells in 20 ml of HBSS containing 0.5% lactalbumin, and the mixture was shaken gently for 20 minutes in the dark at 36°C. The cells were then collected by centrifugation for 5 min-

* Present address: Dept. of Microbiology, University of Chicago, Ill.

TABLE I. Poliovirus and Poliovirus Infective Centers in Infected LLCMK$_2$ Cell Suspensions.

Exp No.	1	2	3	4	5	6	7
Cells/ml in suspension	$10^{4.6}$	$10^{5.0}$	$10^{5.3}$	$10^{5.3}$	$10^{4.8}$	$10^{4.8}$	$10^{4.8}$
Virus recoverable before irradiation (PFU/ml)	$10^{3.7}$	$10^{3.4}$	$10^{2.7}$		$10^{1.9}$	$10^{2.4}$	$10^{1.7}$
Virus recoverable after irradiation (total PFU)	None in 1 ml	None in 135 ml	None in 140 ml	3 or less in 130 ml*	3 or less in 130 ml*	None in 110 ml†	None in 120 ml†
Infective centers/ml after irradiation	$>10^{1.7}$	$10^{1.9}$	$10^{1.2}$	$10^{1.6}$	$10^{1.0}$	$10^{2.4}$	$10^{1.9}$

* Poliovirus was recovered in 1 out of 7 bottles, each containing 19-20 ml of fluid. (The probability that more than 3 PFU was present initially in the positive bottle is less than 0.004.)
† Uncentrifuged suspension of cell debris.

utes at 1500 rpm, washed once, resuspended in 150 ml of HBSS containing 0.5% lactalbumin, and placed in a water-jacketed flask† with a suspended magnetic stirrer. The flask was interposed between 2 General Electric RFL-2 Photoflood lamps at a face-to-face distance of 20 cm. With cold tap water circulating in the jacket and the stirring bar rotating slowly, the vessel was irradiated for 370 seconds. Samples of the suspension were withdrawn before and after the irradiation.

Assay procedures. For determination of infective centers, 0.2-ml samples of a 1:10 dilution of the irradiated cell suspensions were mixed at 45°C with 1.8 ml of 0.45% agar in Earle's salt solution(11) containing 2% calf serum and overlaid on primary rhesus kidney monolayers in sealed 2-oz prescription bottles. After incubation for 2 hours at 36°C, the bottles received an additional overlay of plaque medium containing 3% agar and were maintained at 36°C with observation on the 4th or 5th day to count the plaques.

Samples of the irradiated cell suspension were subjected to 3 cycles of freezing and thawing by alternate immersion in an ethanol-dry ice bath and a 37°C water bath, then were centrifuged for 15 minutes at 1500 rpm to remove cellular debris or, in 2 experiments, were assayed without centrifugation. The supernatant fluids from samples before irradiation were titrated by the plaque method. Supernatant fluids from the irradiated cell suspensions were tested for virus activity by inoculating 20 ml aliquots onto LLCMK$_2$ monolayers in 32-oz prescription bottles. The bottles were observed for 14 days after inoculation and were reported as negative if no cytopathic effect was seen and if the cell lot had been shown to be susceptible to poliovirus infection.

Results. The results of 7 consecutive experiments are summarized in Table I. The washed cell suspensions prior to irradiation contained approximately 10^5 cells per ml and less than 10^4 PFU of virus per ml. Since the cells were originally inoculated at a multiplicity of 1, the virus remaining after washing represented less than 10% of the amount present during adsorption.

The irradiation applied to the cell suspensions had been selected to result in a survival ratio of less than 10^{-6} for photosensitized poliovirus(4). In Experiments 2, 3, 6 and 7, no surviving virus was found in the clarified suspension after freezing and thawing. Traces of virus survived in Experiments 4 and 5, and an unmeasured but small amount may have survived in Experiment 1.

Infective centers surviving the irradiation far outnumbered the recoverable virus particles, although they occurred in only a small proportion (0.01 to 0.1%) of the total number of cells in the suspension. We believe that all of these infective centers were destroyed by the freezing-and-thawing operation, and that they contributed no virus to the system when they were disrupted. To test the possibility that viable virus particles selectively adhered to the cell debris and were removed during centrifugation, we assayed uncentrifuged specimens of cell suspensions after irradiation and disruption, but re-

† Spinner flask No. 3007, 250 ml, Bellco Glass Inc., Vineland, N. J.

covered no virus in either experiment (see Exp. 6 and 7, Table I).

Discussion. There seem to be two possible explanations for these experiments in which we have found infective centers in the absence of virus: 1) the virion, upon entering the cell and losing its protein coat, allows the bound proflavine to diffuse away leaving an intact photoresistant RNA which can later proceed to carry on the course of infection, or 2) the RNA is stabilized against photooxidation during the early stages of infection.

The experiments of Schaffer and Hackett (6) show that poliovirus sensitized with acridine orange and introduced into suspensions of HeLa cells with acridine orange in the medium retains its photosensitivity to a large extent, but their findings would not rule out the presence of some photoresistant eclipsed virus. Wilson and Cooper(3,7) using neutral red and acridine orange as photosensitizers for poliovirus in ERK monolayers found that virus containing either dye lost photosensitivity shortly after penetrating the cell and that this occurred in the continued presence of dye within the cell as well as in its absence. Loss of photosensitivity of the infecting virus thus does not seem to be simply a consequence of disassembly of the particle.

While the present experiments give no support to stabilization of the viral genome as an alternate explanation of photoresistance during eclipse, we note the similarity between our results and those of Fenwick and Pelling(12) and Tershak(13) in which resistance to ultraviolet light developed concurrently with early protein synthesis. Experiments now in progress to test the effects of puromycin and other inhibitors of protein synthesis upon the development of virus-free infective centers may help to clarify the basis for the photoresistant state.

Summary. When suspensions of rhesus monkey kidney cells ($LLCMK_2$) are inoculated with proflavine-photosensitized poliovirus, type 1, incubated 20 minutes at 36°C in the dark, and then irradiated with bright visible light, all detectable virus in the suspension is destroyed, but 0.01 to 0.1% of the cells are capable of developing into infective centers. This finding suggests that RNA from proflavine-photosensitized poliovirus loses its photosensitivity in the early stages of cellular infection, or, alternatively, becomes stabilized shortly after penetration of the cell.

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1. Schaffer, F. L., Virology, 1962, v18, 412.
2. Crowther, D., Melnick, J. L., ibid., 1961, v14, 11.
3. Wilson, J. N., Cooper, P. D., ibid., 1962, v17, 195.
4. Hiatt, C. W., Moore, D., Fed. Proc., 1962, v21, 460.
5. McBride, W. D., Bact. Proc., 1962, 130.
6. Schaffer, F. L., Hackett, A. J., Virology, 1963, v21, 124.
7. Wilson, J. N., Cooper, P. D., ibid., 1963, v21, 135.
8. Morgan, J. F., Morton, H. J., Parker, R. C., Proc. Soc. Exp. Biol. and Med., 1950, v73, 1.
9. Hull, R. N., Cherry, W. R., Tritch, O. J., J. Exp. Med., 1962, v115, 903.
10. Hanks, J. H., Wallace, R. E., Proc. Soc. Exp. Biol. and Med., 1949, v71, 196.
11. Earle, W. R., J. Nat. Cancer Inst., 1943, v4, 165.
12. Fenwick, M. L., Pelling, D., Virology, 1963, v20, 137.
13. Tershak, D. R., ibid., 1964, v23, 1.

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