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Photodynamic Inactivation of Enteroviruses

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ABSTRACT

WALLIS, CRAIG (Baylor University College of Medicine, Houston, Tex.), AND JOSEPH L. MELNICK. Photodynamic inactivation of enteroviruses. J. Bacteriol. **89**:41–46. 1965.—Enteroviruses are usually resistant to photodynamic inactivation, but they can be rendered completely photosensitive to proflavine at *p*H 9 to 10, if they are first purified by filtration through an anion resin. In addition, if enteroviruses are grown in cells maintained in a salt-glucose medium, they can be photosensitized. Of 38 enteroviruses tested, 10 were rendered completely photosensitive to proflavine, or toluidine blue, or both, and the remaining 28 viruses were sensitized, but to a lesser degree. The binding of dye to the virus can be reversed by lowering the *p*H.

Although poliovirus and other enteroviruses are usually resistant to inactivation in the presence of heterotricyclic dyes and white light (Hiatt, 1960; Hiatt et al., 1960), we recently discovered that Type 1 poliovirus could be made photosensitive in neutral red at *p*H 8 if the concentration of organic compounds was kept low (Wallis and Melnick, 1963*a*).

This report describes parameters of photosensitization of 38 enteroviruses. It is noteworthy that they did not all behave alike.

MATERIALS AND METHODS

Monkey kidney (MK) cells. Kidneys from immature rhesus monkeys were trypsinized and grown in Melnick's medium A containing 0.2 mM $AlCl_3$ to suppress adventitious agents (Wallis and Melnick, 1963*b*).

Viruses. Virulent strains of poliovirus were plaque-purified lines of Type 1, Mahoney; Type 2, MEF_1; and Type 3, P24. Attenuated strains were Sabin's plaque-purified lines as used in the oral poliovaccine (type 1, LSc; type 2, P712; and type 3, Leon). Other prototype enteroviruses that grow in MK cells which were also included were 25 echoviruses and 7 coxsackieviruses.

Unless otherwise specified, all enterovirus stocks were grown in Earle's salt solution (ESS), a medium free from organic materials except glucose. Viruses grown in MK cultures maintained in ESS attained titers equal to those of harvests derived from cells maintained with Melnick's medium B.

Virus assays. Enteroviruses were titrated in MK bottle cultures by use of the plaque-forming unit (PFU) method. $MgCl_2$ at 25 mM concentration was incorporated into the overlay medium to expedite plaque counting (Wallis and Melnick, 1962; Wallis, Melnick, and Bianchi, 1962).

Buffers. Sorensen's 0.1 M phosphate buffer (Na_2HPO_4-NaH_2PO_4) was used to attain *p*H levels from 6 to 8; 0.05 M sodium tetraborate for *p*H 9; and 0.05 M tetraborate-NaOH for *p*H 10. Virus samples at *p*H 10 were brought to *p*H 7 to 7.5 with HCl just before plating. Dilutions for assay were made in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (*p*H 7.4).

Purification of viruses by exchange resins. Anion-exchange resin, Dowex 1-X8 (Cl^-), 100 to 200 mesh, was repeatedly washed with 0.85% NaCl until the filtrate reached the same *p*H as the saline used for washing (*p*H 6 to 6.5). To obtain reproducible results, the washing and preparation of exchange resins are of the utmost importance. After the saline washings, columns were washed with phosphate buffer (*p*H 8). The columns, free from excess fluids, were then sterilized by autoclaving, and allowed to cool. Then they were washed with sterile phosphate buffer at *p*H 8 until the filtrate reached *p*H 8. The column was then completely dried by use of nitrogen pressure (2 psi); the nitrogen pressure was then turned off while the gas was still connected to the column. If the pressure is suddenly removed, the resin column will crumble.

Virus harvests were diluted 10-fold in phosphate buffer (*p*H 8), and a 10-ml volume was passed through a packed resin column (10 × 150 mm), using nitrogen pressure (2 psi) to remove organic constituents.

Removal of dye from virus suspensions. Cation-exchange resin 50 W-X4 (H^+), 50 to 100 mesh, was washed as described above. The resin washed in saline was then dispensed and packed in tubes (13 × 100 mm) to a height of 35 mm. They were sterilized by placing them in a boiling-water bath for 15 min. The tubes were cooled, the supernatant saline was removed, and the resin was rewashed with buffer (*p*H 9) until the supernatant fluid

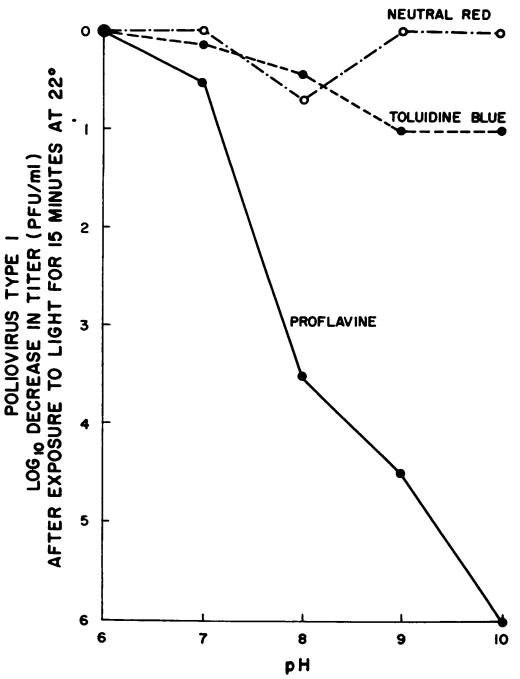

FIG. 1. *Effects of different heterotricyclic dyes and pH on poliovirus.*

attained *p*H 9. After the fluid was removed, 3 ml of virus-dye mixture were placed in each tube, and the tube was gently inverted 8 to 10 times. The tube was then centrifuged at 1,000 rev/min for 2 min, and the supernatant fluid obtained was free from basic dyes. All operations were performed under subdued light. Under these conditions, the basic dyes were completely removed from the virus sample without adsorption of virus to the resin.

Dyes. National Aniline dyes, certified grade (proflavine, neutral red, and Toluidine Blue), were dissolved in distilled water to make a 10^{-3} M stock, and sterilized by boiling. Fresh preparations of dyes were made daily.

Exposure to light. Virus samples were dispensed into new lime-glass tubes (13 × 100 mm) with an 0.8-mm wall. Virus fluids to be kept in the dark were dispensed into amber tubes. Representative samples were exposed 2 in. from two "day-light"-type fluorescent lighting tubes (General Electric, Cool White, 15 watts each). Up to 20 samples could be exposed simultaneously to uniform lighting. At this distance, samples were not significantly affected by the heat transmitted from these cool lights. Immediately after exposure to light, tubes were chilled at 4 C in the dark until titrated (within 20 min).

Tubes were placed in a rack, and backed with aluminum foil for maximal reflection. Virus controls to be kept in the dark in the presence or absence of dye were placed in the rack (in amber test tubes) at locations similar to those of the samples being exposed to light. To minimize the effect of extraneous white light, all virus samples were held in the dark when light was not purposely being applied; experiments were performed and virus was titrated in darkened rooms illuminated by safety red lights.

RESULTS

Effects of different heterotricyclic dyes and pH on Type 1 poliovirus. A previous report (Wallis and Melnick, 1963*a*) showed that poliovirus could be photosensitized at *p*H 8 in 10^{-4} M neutral red by exposing the virus to light for 1 hr. However, with the *p*H level increased to 9 or 10, photosensitization of poliovirus was hardly detectable in neutral red. To select the optimal conditions and heterotricyclic dyes in which to test the enteroviruses, Type 1 poliovirus was used as a model. The virus, derived from a harvest in salt solution, was diluted 10-fold in buffer (*p*H 8) and purified by passage through an anion-resin column. The purified filtrate was diluted 10-fold to contain 10^6 PFU/ml in dye-free buffers, and in final molarities of 10^{-4} M neutral red, 10^{-4} M proflavine, and $10^{-4.5}$ M Toluidine Blue at *p*H levels from 6 to 10. The highest dye concentration which was not toxic to virus in the dark, and through which light could penetrate, was used. Samples were exposed to light at 22 C for 15 min. Control samples in dye-free buffers exposed to light, and virus-dye mixtures kept in the dark (in amber tubes) lost no detectable infectivity at any of the *p*H levels tested, and were not plotted.

Samples in neutral red exposed to light for 15 min gave results (Fig. 1) similar to those previously reported (Wallis and Melnick, 1963*a*). At *p*H 6 and 7, no decrease in titer occurred; at *p*H 8, a decrease of 0.7 $\log_{10}$ in infectivity resulted; at *p*H 9 and 10, no detectable loss of infectivity was manifest. In Toluidine Blue (*p*H 7), a decrease in titer of 0.2 $\log_{10}$ was detected; at *p*H 8, 0.4 $\log_{10}$; and at *p*H 9 and 10, 1 $\log_{10}$. On the other hand, in proflavine (*p*H 7), a decrease in titer of 0.5 $\log_{10}$ was evident; at *p*H 8, 3.5 $\log_{10}$; at *p*H 9, 4.5 $\log_{10}$; and at *p*H 10, complete loss of infectivity occurred (6 $\log_{10}$).

Effect of temperature on photosensitization of poliovirus. Anion-purified virus was diluted 10-fold to contain 10^6 PFU/ml in 10^{-4} M proflavine at *p*H levels from 6 to 10. Preliminary experiments showed that at 35 C, poliovirus was inactivated at *p*H levels above 9.5 in 10^{-4} M proflavine in the dark. However, at 4 and 22 C, samples at *p*H 10 were stable, and thus could be tested at these lower temperatures. Representative samples were equilibrated to 35 C in the

dark, and immediately exposed to light at 35 C for 15 min. Similarly, a series of samples were equilibrated to 22 C and exposed to light at 22 C; other samples were chilled to 4 C and exposed to light at 4 C.

Control samples in 10^{-4} M proflavine in the dark (*p*H 6 to 10), at all temperatures, lost no detectable infectivity, and were not plotted. The samples in proflavine exposed to light at 4 C were not significantly photoinactivated, but at 22 C with *p*H 10 and at 35 C with *p*H 9 the samples were rendered completely inactive (Fig. 2).

We had previously shown that the photosensitization of poliovirus by neutral red at *p*H 8 could be reversed by lowering the *p*H before exposure to light (Wallis and Melnick, 1963*a*). In the present study, virus-neutral red samples, photosensitized at 22 C in the dark at *p*H 9 to 10, became photoresistant if adjusted to *p*H 6 or 7 just before exposure to light. Similarly, virus-dye samples incubated in the dark at 37 C at *p*H 9 for 1 hr, and then exposed to light at 4 C, proved photoresistant.

Effect of light dosage on photosensitivity of poliovirus. Anion-purified poliovirus was used for these experiments to measure the effect of varying the duration of light exposure. The purified virus filtrate was diluted with an equal volume of $10^{-3.7}$ M proflavine to contain 10^8 PFU/ml in 10^{-4} M proflavine. A sample of the virus-proflavine mixture was adjusted to *p*H 9, and a second sample to *p*H 10, with NaOH. Representative samples were then exposed to light for up to 40 min at 22 C (*p*H 10) and at 35 C (*p*H 9). At both temperatures, virus was inactivated exponentially as a first order reaction (Fig. 3).

Effect of oxidized form of proflavine on poliovirus. To determine whether the products resulting from oxidation of proflavine might influence the reaction, a sample of 10^{-4} M proflavine at *p*H 9 was exposed to light for 15 min at 35 C. Purified poliovirus was then diluted to contain 10^6 PFU/ml in (i) the 10^{-4} M proflavine (*p*H 9) solution that had been exposed to light, and (ii) 10^{-4} M proflavine that had been kept in the dark. Representative samples were then exposed to light for 15 min at 35 C, or kept in the dark. Poliovirus kept in the dark was not inactivated to any detectable degree, either in proflavine that had been previously exposed to light, or in freshly prepared proflavine; the titers were $10^{6.1}$ PFU/ml and $10^{6.0}$ PFU/ml, respectively. Virus samples exposed to light in both pretreated and freshly prepared proflavine were completely inactivated.

Effects of tissue culture medium on the photosensitivity of poliovirus. Previous experiments have shown that organic constituents of tissue-

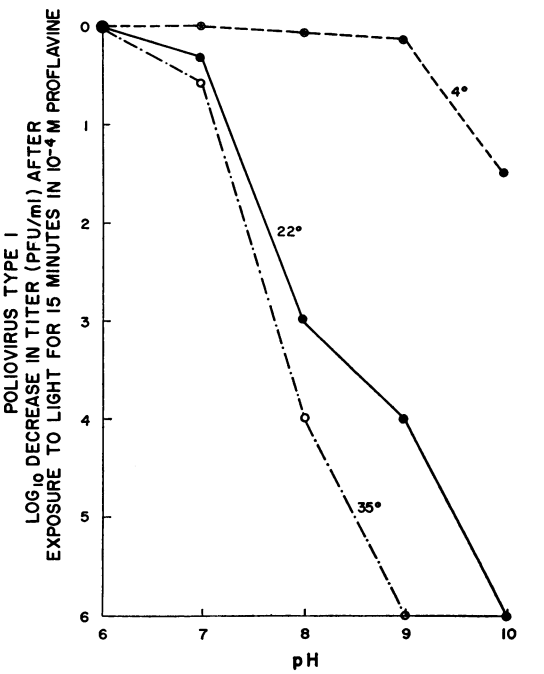

FIG. 2. *Effect of temperature on the photosensitivity of poliovirus.*

culture medium prevented photoinactivation of poliovirus in neutral red at *p*H 8 (Wallis and Melnick, 1963*a*). In view of the new results obtained with proflavine at higher *p*H levels, a re-evaluation was made of the effects of the organic medium. Poliovirus lots grown in a single batch of MK cells, maintained with Melnick's medium or with salt solution, were diluted 10-fold in buffer (*p*H 8), then further diluted 10-fold in dye-free buffer or in 10^{-4} M proflavine (*p*H 10). A sample of the saline harvest diluted 10-fold at *p*H 8 was purified by passage through an anion resin, and then diluted 10-fold in dye-free buffer and proflavine, as described above. Samples were exposed to light for 15 min at 22 C (Table 1). Dye-free samples exposed to light and samples in proflavine kept in the dark were not inactivated. The harvest in Melnick's medium exposed to light in proflavine lost 1 $\log_{10}$ in titer, and the saline harvest in proflavine decreased in titer 3 $\log_{10}$, whereas the purified virus in proflavine was completely inactivated (6.0 $\log_{10}$).

Photosensitivity of other polioviruses. Attenuated and virulent poliovirus strains, representing the three immunological types, were grown in MK cells maintained with ESS. Supernatant fluids of each clarified virus harvest were diluted 10-fold in dye-free buffer and in 10^{-4} M proflavine (*p*H 10), and in $10^{-4.5}$ M Toluidine Blue (*p*H 10).

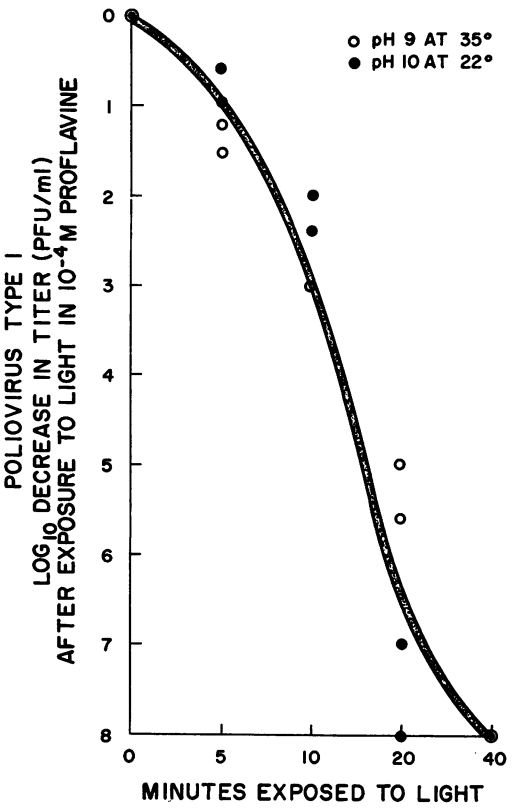

FIG. 3. *Effect of exposure time on photosensitivity of poliovirus.*

TABLE 1. *Effects of tissue culture medium on the photosensitivity of Type 1 poliovirus*

Source of virus	Dye-free buffer		10^{-4} M proflavine	
	Light	Dark	Light	Dark
Harvest in Melnick's medium	6.2*	6.3	5.3	6.4
Harvest in Earle's saline	6.2	6.1	3.0	6.0
Purified virus	6.2	6.2	0.0	6.0

* Results expressed as $\log_{10}$ virus titer (plaque-forming units per milliliter).

Representative samples were exposed to light at 22 C for 15 min. The results (Table 2) indicated that all the polioviruses tested were photosensitized by proflavine to a greater degree than by Toluidine Blue. Toluidine Blue at 10^{-4} M afforded less photosensitivlty than at $10^{-4.5}$ M, because light could not penetrate the sample freely. At 10^{-5} M Toluidine Blue, dye concentration was insufficient for photosensitization.

Photosensitivity of echo- and coxsackieviruses. Viruses were grown and harvested in salt solution, then diluted 10-fold in dye-free buffer, or in 10^{-4} M proflavine and $10^{-4.5}$ M Toluidine Blue (*p*H 10). Samples were not anion-purified, but were tested as described above for polioviruses. The results are described in Tables 3 and 4.

Of the 25 echoviruses tested, only seven strains (types 2, 18, 20, 22, 23, 24, and 30) were completely photosensitized by both proflavine and Toluidine Blue. Types 11, 26, and 32 were rendered photosensitive only with proflavine, and type 3 only with Toluidine Blue. Of the seven coxsackieviruses tested, none was rendered completely photosensitive; however, with every enterovirus tested, some degree of photoinactivation occurred.

Reversibility of enterovirus-dye union. All enteroviruses which were fully photosensitive to proflavine, or Toluidine Blue, or both, were tested to determine whether the virus-dye complex was a tightly bound union. Samples of enteroviruses in dyes were incubated at 37 C for 1 hr in the dark, and the dye was then removed from the virus suspension by cation resin treatment. The water-clear virus suspensions were then exposed to light for 15 min. Of the 10 viruses tested in proflavine (poliovirus Type 1; echovirus types 2, 18, 20, 22, 23, 24, 26, 30, and 32) and the one tested in Toluidine Blue (echovirus type 3), none was found to be inactivated. Thus, in all cases, the dye could be recovered from the virus, leaving a photoresistant particle.

DISCUSSION

It is evident that enteroviruses not only differ from other virus groups in their photosensitivity, but also differ among themselves. Viruses belong-

TABLE 2. *Photosensitivity of polioviruses (saline harvests, unpurified)*

Poliovirus	Dye-free buffer		10^{-4} M proflavine		$10^{-4.5}$ M Toluidine Blue	
	Light	Dark	Light	Dark	Light	Dark
Attenuated						
Type 1	6.3*	6.1	3.0	6.4	5.6	6.2
Type 2	5.5	5.5	4.0	5.3	5.1	5.4
Type 3	6.0	6.0	2.0	6.2	5.0	5.9
Virulent						
Type 1	7.2	7.4	3.5	7.0	6.0	7.0
Type 2	6.8	6.8	3.8	6.8	6.0	6.5
Type 3	7.0	7.0	3.0	7.0	6.2	6.8

* Results expressed as $\log_{10}$ virus titer (plaque-forming units per milliliter).

ing to the pox-, herpes-, reo-, myxo-, and papovavirus groups can be irreversibly photosensitized in the presence of tissue-culture organic components (Wallis and Melnick, 1964). Thus, free dye can be removed from the virus suspension by cation resins, and the resulting water-clear virus suspension remains photosensitive. On the other hand, enteroviruses can be photosensitized only after purification through anion resins to remove organic medium, or by growing virus in a simple salt-glucose medium. Even under these conditions, the effects were reversible; after removal of dye, the virus suspension became photoresistant. The binding of virus to dye can also be reversed by lowering *p*H or temperature just before exposure to light. Of 38 enteroviruses belonging to the polio, coxsackie, and echo subgroups, grown in salt-glucose medium and tested without further purification, all became photosensitized to some degree, but only 10 types, all of them echoviruses, were rendered completely photosensitive by proflavine or Toluidine Blue, or both.

TABLE 3. *Photosensitivity of echoviruses (saline harvests, unpurified)*

Prototype[a] echovirus	Dye-free buffer		10^{-4} M proflavine		$10^{-4.5}$ M Toluidine	
	Light	Dark	Light	Dark	Light	Dark
1	6.5*	6.5	4.3	6.3	3.6	6.6
2	6.5	6.5	0.0	6.2	0.0	6.3
	6.3	6.5	0.0	6.5	0.0	6.5
3	5.3	5.1	4.5	5.3	0.0	5.1
	5.0	5.0	4.0	5.1	0.0	5.0
4	4.0	4.2	3.0	4.2	3.8	4.1
5	6.2	6.4	4.4	6.6	3.4	6.1
6	6.6	6.5	4.0	6.5	4.1	6.8
7	6.7	6.8	5.8	7.0	4.7	6.7
9	6.7	6.7	3.7	6.5	3.7	6.4
11	4.5	4.6	0.0	4.7	2.7	4.8
	4.2	4.5	0.0	4.5	2.0	4.5
12	6.2	6.4	4.0	6.5	5.4	6.5
13	5.0	5.2	4.0	5.0	4.4	5.2
14	5.1	5.0	2.0	5.0	3.0	5.0
16	5.0	5.1	2.5	5.0	3.0	5.0
17	6.7	6.7	3.5	6.5	4.0	6.4
18	3.5	3.5	0.0	3.3	0.0	3.3
	3.2	3.5	0.0	3.1	0.0	3.2
20	4.5	4.7	0.0	4.4	0.0	4.3
22	6.0	6.2	0.0	6.0	0.0	5.9
23	4.9	4.8	0.0	4.9	0.0	4.7
24	4.5	4.5	0.0	4.1	0.0	4.3
25	5.7	5.6	2.7	5.4	3.0	5.1
26	5.4	5.4	0.0	5.3	2.0	5.0
27	4.6	4.5	2.6	4.4	2.4	4.2
29	4.5	4.5	1.5	4.1	1.6	4.4
30	4.0	4.0	0.0	4.0	0.0	3.9
32	4.2	4.0	0.0	3.7	2.2	4.0

* Results expressed as $\log_{10}$ virus titer (plaque-forming units per milliliter).

TABLE 4. *Photosensitivity of coxsackieviruses (saline harvests, unpurified)*

Prototype Coxsackie virus	Dye-free buffer		10^{-4} M proflavine		$10^{-4.5}$ M Toluidine Blue	
	Light	Dark	Light	Dark	Light	Dark
A9	7.2*	7.0	6.2	7.0	6.0	6.9
B1	5.2	5.4	4.0	5.1	4.0	5.0
B2	6.4	6.7	4.4	6.5	6.4	6.7
B3	6.0	6.1	4.5	6.0	5.5	6.0
B4	5.8	5.9	3.8	5.5	5.1	5.5
B5	6.0	6.2	3.0	6.0	5.8	6.0
B6	6.2	6.2	5.7	6.0	5.0	6.3

* Results expressed as $\log_{10}$ virus titer (plaque-forming units per milliliter).

Three attenuated and three virulent polioviruses reacted similarly to light in the presence of dyes. All were photosensitized with proflavine to a greater degree than with Toluidine Blue. In contrast, echovirus types 2, 18, 20, 22, 23, 24, and 30 were completely photosensitized by both proflavine and Toluidine Blue. Types 11, 26, and 32 were completely photosensitized by proflavine, and only partially by Toluidine Blue. Type 3 was completely sensitized by Toluidine Blue and partially by proflavine. The remaining echo- and coxsackieviruses were all partially photosensitized by both dyes.

A possibility that toxic products of proflavine oxidation might be the inactivating factor with enteroviruses, rather than the action of light on the virus-dye complex, was ruled out. Proflavine treated with light, and then added to the virus and kept in the dark, failed to reduce the infectivity of the virus. This sample when exposed to light yielded results similar to those with untreated proflavine.

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