THE INACTIVATION OF ENTEROVIRUS INFECTIVITY BY THE SULFHYDRYL REAGENT *p*-CHLOROMERCURIBENZOATE*

BY PURNELL W. CHOPPIN, M.D., AND LENNART PHILIPSON,‡ M.D.

(From The Rockefeller Institute)

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In a previous communication (1) it was reported that a number of sulfhydryl reagents inactivated the hemagglutinating activity of enteroviruses. Detailed studies with *p*-chloromercuribenzoate (PCMB) showed that treated virus failed to adsorb to erythrocytes. Inactivation of hemagglutinating activity by mercaptide-forming sulfhydryl reagents was reversed by thiol compounds. These and other results suggested that sulfhydryl groups are involved in the adsorption of enteroviruses to erythrocytes.

In preliminary experiments (1), it was also found that a number of sulfhydryl reagents decreased the infectivity of several enteroviruses. In the present communication, the results of further studies on the effects of a sulfhydryl reagent on the infectivity of enteroviruses are presented. The inactivation of infectivity of enteroviruses by PCMB, and the effect of various buffers and divalent cations on the inactivation are described. The reversal by reduced glutathione of the inactivation of infectivity by PCMB is reported. It is shown that under certain conditions, PCMB prevents the adsorption of some enteroviruses to monlayers of host cells in culture.

Materials and Methods

Viruses.—The following enteroviruses were used: ECHO type 7 (Wallace strain), ECHO 9 (A.B. strain) (2), Coxsackie B3 (a hemagglutinating strain obtained from Dr. L. Rosen of the National Institutes of Health), and polio 2 (MEF1 strain). The propagation and storage of these viruses has been previously described (1). "Virus suspension" refers to virus-containing supernatant fluid removed from cell cultures without prior freezing and thawing of cultures. Debris was removed from such suspensions by low speed centrifugation. In some instances, virus was partially purified by differential centrifugation by the following procedure: (*a*) virus suspension centrifuged at 10,000 *g* for 10 minutes, and sediment discarded; (*b*) supernate centrifuged at 78,000 *g* for 4 hours, and supernate discarded; (*c*) sediment resuspended and centrifuged at 10,000 *g* for 10 minutes, and sediment discarded; (*d*) supernate centrifuged at 105,000 *g* for 2 hours, and supernate discarded; (*e*) sediment resuspended in

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‡ Sophie Fricke Fellow of the Royal Swedish Academy of Science in The Rockefeller Institute.

phosphate-buffered saline or other buffers. *Diluents.*—The solution designated phosphate-buffered saline (PBS) (3) contained 0.0081 M KH_2PO_4, 0.0015 M Na_2HPO_4, 0.137 M NaCl, 0.0027 M KCl, 0.0009 M $CaCl_2$ and 0.0005 M $MgCl_2 \cdot 6H_2O$. The solution designated tris-buffered saline (TBS), pH 7.2, contained 0.025 M tris(hydroxymethyl)aminomethane, 0.137 M NaCl, 0.005 M KCl, 0.0004 M $Na_2HPO_4 \cdot 7H_2O$, 0.0005 M $MgCl_2$, and 0.0009 M $CaCl_2 \cdot 2H_2O$. In some experiments, phosphate- or tris-buffered solutions were used which contained only NaCl as the additional salt. The solution designated barbital buffer consisted of 0.15 M NaCl buffered at pH 7.2 with 0.01 M barbital, and the solution designated borate buffer consisted of 0.15 M NaCl buffered at pH 7.0 with 0.02 M borate.

Reagents.—*p*-Chloromercuribenzoic acid, obtained from K & K Laboratories, Inc., Jamaica, New York, was dissolved in 1 N NaOH. The solution was diluted to 10^{-3} M in deionized distilled water, and the pH adjusted to 7.2 with 1 N HCl. Stock solutions were held at 4°C. for periods not exceeding 3 weeks. Reduced glutathione, obtained from Mann Research Laboratories, Inc., New York, was dissolved in water and the pH adjusted to 7.2 with 0.1 N NaOH. Reduced glutathione was freshly prepared for each experiment. These reagents were diluted to the required concentrations in the appropriate buffers for each experiment.

Cell Cultures.—Monkey kidney cells were obtained as previously described (1), and grown in plastic culture dishes[1] 6 cm. in diameter in Hanks' solution (4) with 2 per cent calf serum and 0.5 per cent lactalbumin hydrolysate. KB cells were obtained from Microbiological Associates, Bethesda, Maryland, and grown in continuous culture in Eagle's basal medium (5) with 10 per cent human serum. Plastic culture dishes were seeded with 1.5×10^6 cells per dish. All cell cultures were incubated at 37°C. in a humidified atmosphere of 5 per cent CO_2 in air.

Plaque Assay.—Plastic dishes with confluent sheets of cells were washed twice with 5 ml. of the appropriate buffer, and inoculated with 0.5 ml. volumes of serial tenfold dilutions of virus. Two to four dishes were inoculated with each dilution. After an adsorption period of 30 minutes at 37°C., the cultures were washed twice with the appropriate buffer, and the overlay was added. With monkey kidney cells, the overlay consisted of equal volumes of 1.9 per cent ionagar and two times concentrated Hanks' solution (4) with 0.2 per cent bovine albumin and 0.2 per cent yeast extract. With KB cells, the overlay consisted of equal volumes of 1.9 per cent ionagar and two times concentrated Eagle's basal medium (5) with 4 per cent chicken serum and 2 per cent calf serum. After the overlay was added the cultures were held in the CO_2 incubator for 2 days, and then a second overlay containing 0.0025 per cent neutral red was added. Plaques were counted after 3 days of incubation. The amount of infective virus was expressed in plaque-forming units (PFU).

EXPERIMENTAL

Effect of p-Chloromercuribenzoate (PCMB) on Infectivity of Enteroviruses.—It was reported previously (1) that several sulfhydryl reagents, including PCMB, iodoacetamide, and *N*-ethylmaleimide, inactivated the infectivity of some enteroviruses. PCMB was selected for further study, and its effects on the infectivity of ECHO 7, ECHO 9, Coxsackie B3, and polio 2 viruses were investigated by the plaque technique.

Virus suspensions were diluted 1:10 or 1:100 in PBS and mixed with equal volumes of PCMB in PBS. The final concentration of PCMB in the reaction mixture was 10^{-4} M. Controls consisted of virus in PBS. The mixtures were incubated at 37°C. for 30 minutes, and

[1] TCPD6015, Falcon Plastics Company, Los Angeles, California.

infective virus was then assayed on monkey kidney cell monolayers by the plaque technique. In early experiments excess PCMB was removed by dialysis before infectivity was assayed; however, it was found that the same results were obtained without dialysis, because in the titration procedure PCMB was diluted out below the level of effective concentrations. Therefore, in subsequent experiments, dialysis was not carried out.

As can be seen in Table I, the greatest degree of inactivation, a 1000-fold reduction in infectivity, was obtained with ECHO 7 virus. Intermediate inactivation was obtained with ECHO 9 and Coxsackie B3; and slight, but reproducible, inactivation with polio 2. The values shown in Table I are the means of three or four experiments with the same pool of each virus. When

TABLE I

Effect of p-Chloromercuribenzoate (PCMB) on the Infectivity of Enteroviruses

Virus	Infective virus, PFU per ml.		Fold reduction in PFU
	Control*	PCMB 1×10^{-4} M*	
ECHO 7	4.1×10^7	4.1×10^4	1000
ECHO 9	7.0×10^7	9.0×10^5	78
Coxsackie B3	3.1×10^6	1.2×10^4	260
Polio 2	1.4×10^7	2.8×10^6	5

* Buffer: phosphate-buffered saline (PBS).

different pools of virus were tested there was considerable variation in the extent of inactivation, but significant inactivation was always obtained. As will be shown below, differences in the extent or rate of inactivation by PCMB were observed which were dependent on the purity of the virus preparation and the buffer in which the reaction is carried out.

Kinetics of Inactivation of Enteroviruses by PCMB.—It was shown previously that the rate of inactivation of enterovirus hemagglutinin by sulfhydryl reagents is rapid (1). The kinetics of inactivation of the infectivity of ECHO 7, ECHO 9, Coxsackie B3, and polio 2 viruses by PCMB were investigated.

Virus suspensions were diluted 3-fold in PBS and mixed with equal volumes of PCMB diluted to give a final concentration of 5×10^{-5} M. Controls consisted of virus in PBS. Mixtures were incubated at 37°C. in a waterbath, and at intervals aliquots were removed and immediately immersed in an ice bath. Infective virus was then assayed by the plaque technique on monolayers of KB cells with polio 2 virus, and monkey kidney cells with all other viruses.

Fig. 1 shows the inactivation curves obtained in these experiments. With ECHO 7, ECHO 9, and Coxsackie B3 viruses, inactivation was rapid, 50 per cent inactivation occurring within 1 minute with ECHO 7, and within 2 minutes with ECHO 9 and Coxsackie B3. With these three viruses, the first portion of the curves resembled that of a first order reaction, however with ECHO 7

and ECHO 9 there was a leveling off which was more pronounced with ECHO 9. With Coxsackie B3, the curve resembled that of a first order reaction for the duration of the experiment. With polio 2 virus, a total inactivation of only 50 per cent was obtained, and thus the shape of the curve is difficult to interpret.

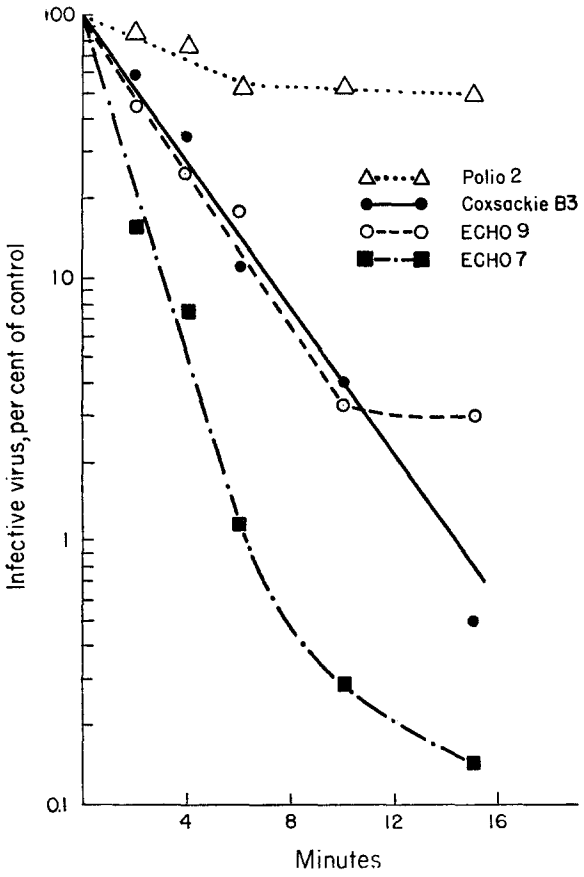

FIG. 1. Kinetics of inactivation of enterovirus infectivity by *p*-chloromercuribenzoate (PCMB). The final concentration of PCMB was 5×10^{-5} M. Virus was treated at 37°C. in phosphate-buffered saline (PBS).

Effect of Different Buffers on the Inactivation of Infectivity of Enteroviruses by PCMB.—The results of a number of inactivation experiments with PCMB suggested that the extent of inactivation was influenced by factors other than the virus used. Virus contained in supernatant fluids collected after disruption of infected cells by freezing and thawing was not inactivated by PCMB to the same extent as virus contained in supernatant fluids removed

from cultures prior to disruption of cells. This finding may be due to the release from the disrupted cells of materials, *e.g.* proteins, which can react with PCMB. However, preliminary experiments indicated that the buffer in which the reaction between PCMB and virus was carried out also affected the inactivation of the virus. The effect of various buffers on the inactivation of the infectivity of enteroviruses by PCMB was therefore investigated.

Virus suspensions were centrifuged at 105,000 *g* for 2 hours and resuspended in 0.15 M NaCl. Aliquots were then diluted 1:100 in either phosphate-buffered saline (PBS) or tris-buffered saline (TBS). Equal volumes of virus dilutions and PCMB, diluted in the appropriate buffer to give a final concentration of 5×10^{-5} M, were mixed and incubated at 37°C. for 16 minutes. Controls consisted of virus in appropriate buffers. Serial 10-fold dilutions were then made in the appropriate buffers, and infective virus assayed by the plaque technique.

TABLE II

Effect of Phosphate and Tris Buffers on the Inactivation of Enteroviruses by p-Chloromercuribenzoate (PCMB)

Virus	Infective virus, PFU per ml.					
	Phosphate-buffered saline (PBS)			Tris-buffered saline (TBS)		
	Control	PCMB 5×10^{-5} M	Fold reduction in PFU	Control	PCMB 5×10^{-5} M	Fold reduction in PFU
ECHO 7	1.1×10^{7}	1.0×10^{3}	10,000	6.1×10^{6}	3.1×10^{5}	20
ECHO 9	6.1×10^{6}	2.0×10^{3}	3,100	4.9×10^{6}	1.3×10^{6}	3.8
Coxsackie B3	4.6×10^{6}	$<5.0 \times 10^{2}$	>9,200	5.0×10^{6}	4.7×10^{4}	110
Polio 2	6.2×10^{6}	1.0×10^{6}	6.2	7.0×10^{6}	2.5×10^{6}	2.8

The results of experiments with four enteroviruses, using PBS or TBS as a diluent, are shown in Table II. As can be seen, the inactivating effect of PCMB was regularly greater in PBS than in TBS. It can also be seen that more inactivation in PBS occurred in these experiments than in the experiments shown in Table I. The greater effect in the later experiments was presumably due to the fact that the viruses used had been partially purified by differential centrifugation.

To investigate further the above finding of greater inactivation by PCMB in PBS than in TBS, the effect on inactivation of the addition of phosphate to a tris buffer was determined.

A buffer was prepared containing 0.01 M tris, 0.15 M NaCl, and 0.001 M $CaCl_2$, and additional solutions were prepared by adding to this buffer KH_2PO_4 to a concentration of from 10^{-6} to 10^{-3} M. The pH of the solutions varied from 7.2 to 7.0. ECHO 7 virus, partially purified by differential centrifugation, was diluted in each of the solutions, and mixed with equal volumes of PCMB diluted in the appropriate solutions to give a final concentration of 1×10^{-4}

M. The mixtures were held at 37°C. for 10 minutes, and the virus was then assayed by the plaque technique.

The results of these experiments are shown in Table III. As can be seen, the extent of inactivation increased with increasing phosphate concentration, indicating that inactivation of ECHO 7 virus by PCMB was enhanced by the presence of phosphate.

Kinetics of Inactivation of ECHO 7 virus by PCMB in Different Buffers.—The kinetic features of inactivation of ECHO 7 virus in NaCl solutions buffered with either tris or phosphate were compared.

TABLE III

Effect of the Addition of Phosphate to Tris Buffer on the Inactivation of ECHO 7 Virus by p-Chloromercuribenzoate (PCMB)

Treatment of Virus		Infective virus, PFU per ml.	Fold reduction in PFU
	Concentration of KH_2PO_4		
Control	0	1.2×10^7	—
PCMB, 1×10^{-4} M	0	1.3×10^5	92
PCMB, 1×10^{-4} M	10^{-6} M	1.1×10^5	110
PCMB, 1×10^{-4} M	10^{-5} M	9.4×10^4	130
PCMB, 1×10^{-4} M	10^{-4} M	6.6×10^4	180
PCMB, 1×10^{-4} M	10^{-3} M	1.8×10^4	670

The buffers used were 0.15 M NaCl buffered at pH 7.2 with 0.01 M tris or with 0.01 M phosphate. ECHO 7 virus was diluted 1:100 in each buffer, and then mixed with equal volumes of PCMB diluted in the appropriate buffers to give a final concentration of 5×10^{-5} M. Controls consisted of virus in appropriate buffers not containing PCMB. Mixtures were held at 37°C., and at intervals aliquots were removed, immersed in an ice bath, and then serially diluted in the appropriate buffers and assayed by the plaque technique.

As can be seen in Fig. 2, in phosphate buffer there was initially very rapid inactivation of infective virus. This was followed by reappearance of some of the infectivity. In tris buffer there was less inactivation and no evidence of return of infectivity on further incubation. Similar results were also obtained with virus which had been purified by differential centrifugation and by chromatography on a DEAE cellulose column (6).

Kinetic experiments were also carried out in 0.15 M NaCl buffered at pH 7.2 with 0.01 M barbital, and in 0.15 M NaCl buffered at pH 7.0 with 0.02 M borate. In borate buffer infectivity was inactivated as rapidly as in phosphate buffer, and a very slight return of infectivity was observed on further incubation. In barbital buffer inactivation was very slow, 88 per cent in 16 minutes. This rate is slower than that obtained in tris buffer.

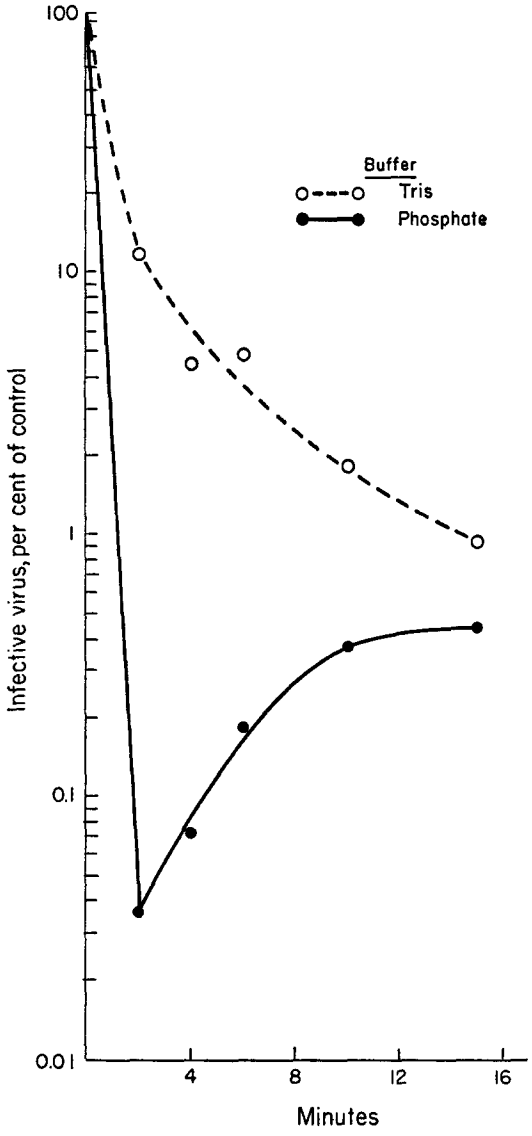

FIG. 2. Kinetics of inactivation of ECHO 7 virus infectivity by *p*-chloromercuribenzoate (PCMB) at pH 7.2 in 0.15 M NaCl buffered with 0.01 M tris(hydroxymethyl)aminomethane (tris) or 0.01 M phosphate. No divalent cations were added. Final concentration of PCMB was 5×10^{-5} M. Virus was treated at 37°C.

These results indicate that the rate of inactivation of the infectivity of ECHO 7 virus by PCMB is dependent on the buffer in which the reaction is carried out. It has been shown previously (7, 8) that the rate of reaction of sulfhydryl groups of proteins with PCMB is markedly affected by the presence of certain anions. It is thought (8) that in reactions of phenylmercuric compounds with sulfhydryl groups, the reactive species is most likely not the phenylmercuric ion, but a derivative formed with anions such as buffer anions present in large molar excess over the mercurial added.

Effect of Calcium and Magnesium on the Rate of Inactivation of ECHO 7 Virus by PCMB.—The above results with different buffers agree well with those summarized in Tables II and III showing that phosphate ions enhance the inactivating effect of PCMB on viral infectivity. However, the curve in Fig. 2 depicting inactivation of ECHO 7 virus in phosphate buffer without divalent cations differs from that in Fig. 1. In the experiments sumarized in Fig. 1, PBS, which contains both calcium and magnesium, was used, and no return of infectivity occurred on prolonged incubation. This suggested that the divalent cations might have prevented the return of infectivity, and this possibility was investigated in experiments with ECHO 7 virus and phosphate and tris buffers to which calcium or magnesium was added.

To aliquots of each of the buffers used in the experiments shown in Fig. 2, *i.e.* 0.15 M NaCl buffered at pH 7.2 with 0.01 M tris or with 0.01 M phosphate, $CaCl_2$ or $MgCl_2$ was added to a concentration of 0.001 M. ECHO 7 virus, partially purified by differential centrifugation, was diluted 1:100 in each of the above buffers. The diluted virus suspensions were mixed with equal volumes of PCMB diluted in the appropriate buffers to give a final concentration of 1×10^{-4} M. Controls consisted of virus in appropriate buffers not containing PCMB. The mixtures were held at 37°C., and at intervals aliquots were removed, chilled in an ice bath, serially diluted, and assayed by the plaque technique.

The results of these experiments are shown in Fig. 3. In phosphate buffer containing either calcium or magnesium there was rapid inactivation by PCMB and no return of infectivity. The curves thus obtained resemble those in Fig. 1. In tris buffer with either calcium or magnesium there was less inactivation by PCMB than in phosphate buffer, and no return of infectivity.

These results indicate that both calcium and magnesium can prevent the return of infectivity seen in the absence of these divalent cations in phosphate buffer. They also confirm the greater inactivation by PCMB in phosphate buffer as compared to tris buffer regardless of the presence or absence of magnesium or calcium.

Effect of Different Buffers on Inactivation of Hemagglutinating Activity of Enteroviruses by PCMB.—Because of the above demonstration of the effect of different buffers on the inactivation of infectivity by PCMB, the effect of various buffers on the inactivation by PCMB of the hemagglutinating activity of ECHO 7 virus was investigated.

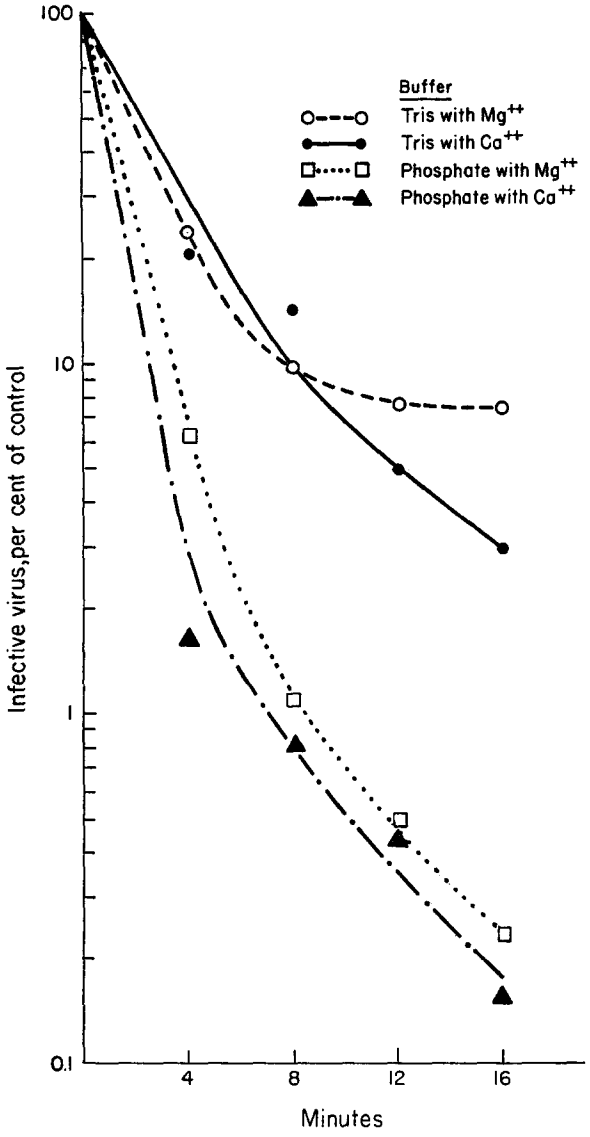

FIG. 3. Kinetics of inactivation of ECHO 7 virus infectivity by *p*-chloromercuribenzoate (PCMB) at pH 7.2 in 0.15 M NaCl buffered with 0.01 M tris(hydroxymethyl)aminomethane (tris) or 0.01 M phosphate, with 0.001 M $CaCl_2$ or $MgCl_2$. Final concentration of PCMB was 1×10^{-4} M. Virus was treated at 37°C.

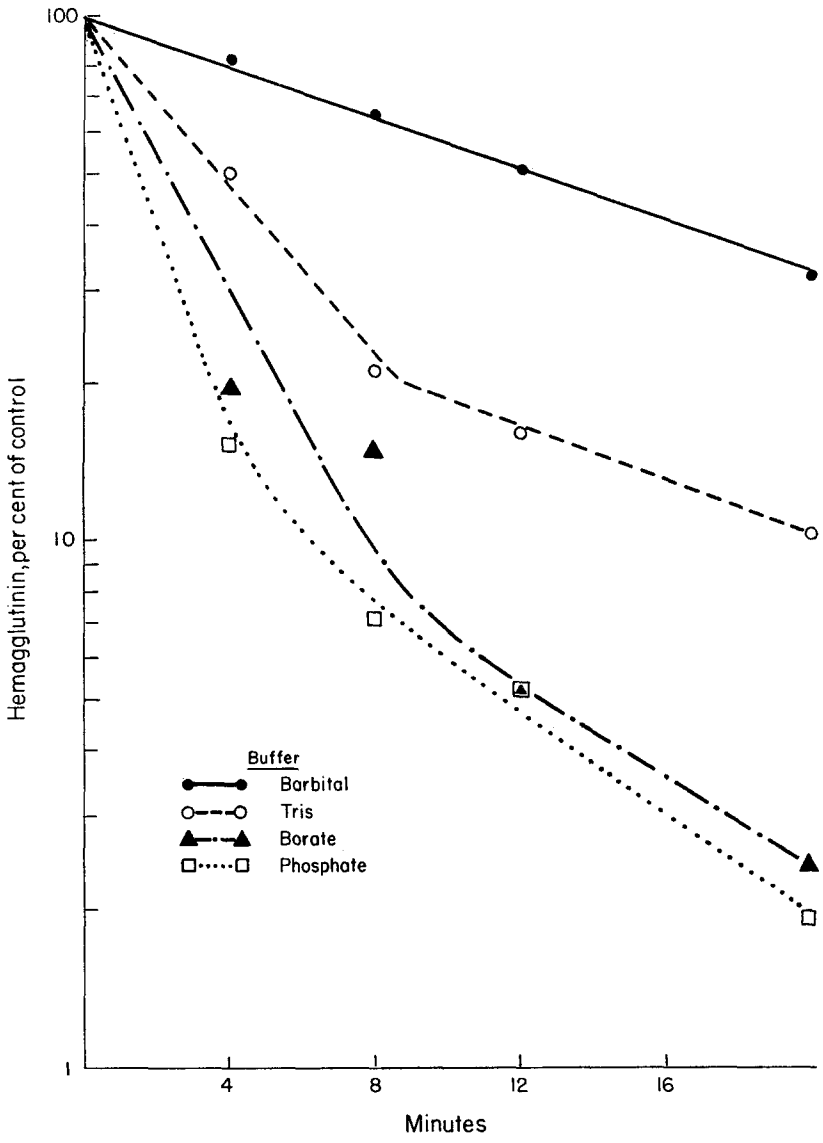

FIG. 4. Kinetics of inactivation of ECHO 7 virus hemagglutinin by *p*-chloromercuribenzoate (PCMB) in the following buffers: 0.01 M barbital, pH 7.2; 0.01 M tris(hydroxymethyl) aminomethane (tris), pH 7.2; 0.01 M phosphate, pH 7.2; 0.02 M borate, pH 7.0. All buffer solutions contained 0.15 M NaCl. Final concentration of PCMB was 2×10^{-4} M. Virus was treated at 37°C.

ECHO 7 virus was partially purified by differential centrifugation and suspended in various buffers. The buffers employed were 0.01 M phosphate, pH 7.2; 0.01 M tris, pH 7.2; 0.01 M barbital, pH 7.2; and 0.02 M borate, pH 7.0. All of the buffer solutions contained 0.15 M NaCl. Equal volumes of virus suspensions and PCMB, diluted in the appropriate buffer to give a final concentration of 2×10^{-4} M, were mixed and incubated at 37°C. At intervals aliquots were removed, immediately chilled in an ice bath, and assayed in hemagglutination titrations. The titrations were done by the method previously described (1) with the exception that all serial dilutions were made in 0.15 M NaCl without buffer, and human O erythrocytes were also suspended in the same solution.

The results of a typical experiment are shown in Fig. 4. The most rapid inactivation of the hemagglutinating activity of ECHO 7 virus was obtained in phosphate and borate buffers. A slower rate was obtained in tris buffer, and a considerably slower rate in barbital buffer. Similar results were obtained in numerous separate experiments. These results are in agreement with those obtained in the experiments on inactivation of infectivity.

The decreased inactivation by PCMB in barbital buffer may explain the previously reported (1) finding that the inactivation of hemagglutinating activity by PCMB was less complete at pH 8 or 9 than at lower pH. In those experiments the inactivation at pH 8 or 9 was carried out in barbital buffers.

Restoration by Reduced Glutathione of the Infectivity of PCMB-treated Enteroviruses.—An important property of mercaptides, formed by the combination of sulfhydryl groups and heavy metals, is the reversal of the reaction by thiol compounds (9). It was previously shown that the inactivation of the hemagglutinating activity of enteroviruses by mercaptide-forming sulfhydryl reagents could be reversed by thiol compounds (1). The ability of reduced glutathione to reverse the inactivation of the infectivity of enteroviruses by PCMB was investigated.

The buffer used was TBS, which contained calcium and magnesium. Spontaneous return of infectivity has not been observed in this buffer. Virus suspensions, diluted 10^3- to 10^5-fold in TBS, were mixed with equal volumes of PCMB diluted in TBS to give a final concentration of 1×10^{-4} M. Controls consisted of virus in TBS. Mixtures were held at room temperature for 10 minutes, and then dialyzed overnight at 4°C. against TBS. The infective virus in untreated controls and in PCMB-treated samples was determined by the plaque technique before the addition of reduced glutathione. The dialyzed samples were then mixed with equal volumes of reduced glutathione diluted in TBS to give a final concentration of 5×10^{-3} M. Mixtures were held at room temperature and at intervals aliquots were taken, diluted in TBS, and assayed for infective virus by the plaque technique.

Fig. 5 shows the inactivation of infectivity of ECHO 7, Coxsackie B3, and ECHO 9 viruses by PCMB, and reactivation by reduced glutathione. As can be seen, approximately 96 per cent inactivation of ECHO 7 virus infectivity had occurred after treatment with PCMB at room temperature for 10 minutes. Reduced glutathione caused 85 per cent reactivation of infectivity within 15 minutes. With Coxsackie B3 virus, more than 98 per cent inactivation and

50 per cent reactivation were observed. With ECHO 9 virus there was only slight reactivation of infectivity by reduced glutathione. In fact, during the 2 minute interval which followed the addition of glutathione, there was a

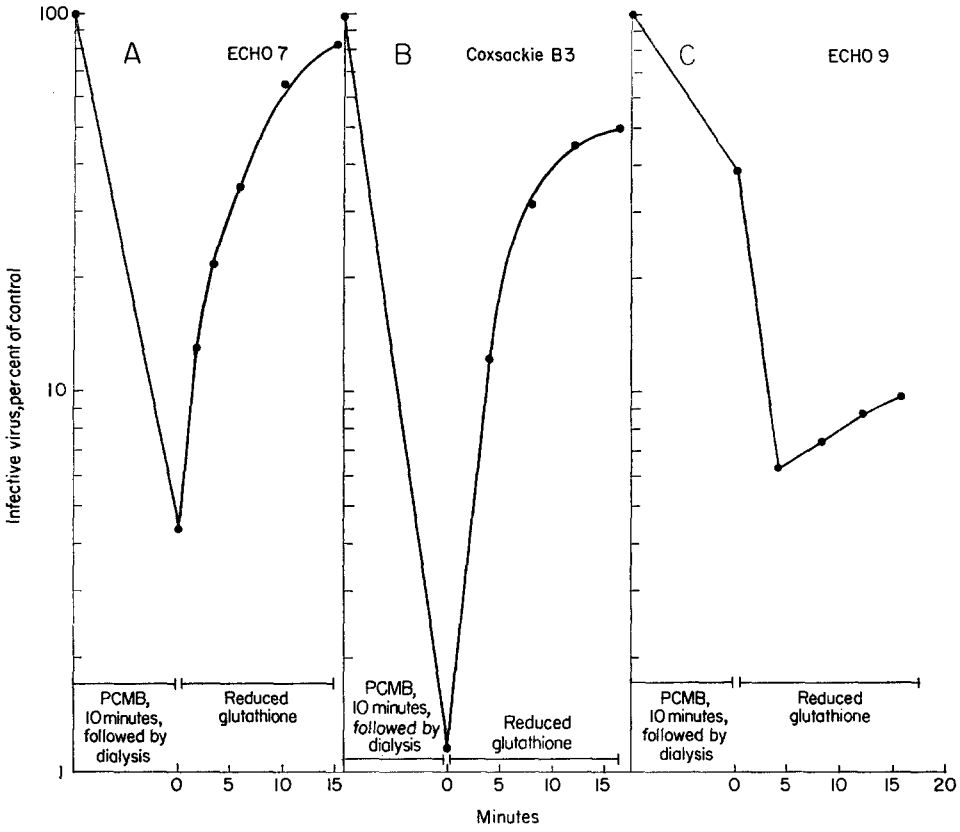

FIG. 5. Reactivation by reduced glutathione of enterovirus infectivity inactivated by *p*-chloromercuribenzoate (PCMB). Virus was treated at room temperature for 10 minutes with PCMB at a final concentration of 1×10^{-4} M in tris(hydroxymethyl)aminomethane-buffered saline (TBS). After dialysis at 4°C. against TBS, the virus was treated at room temperature with reduced glutathione at a final concentration of 5×10^{-3} M.

further decrease in infectivity. The explanation for this finding is not known at the present time.

The extent of inactivation of polio 2 virus by PCMB under the conditions of these experiments was too small to permit adequate demonstration of reactivation.

In experiments of the same design in which inactivation of ECHO 7 or Coxsackie B3 virus by PCMB was carried out in PBS instead of TBS, greater

inactivation was observed, and there was less reactivation by reduced glutathione. Similar results were obtained when treatment with PCMB was carried out at 37°C. rather than at room temperature, and when treatment was prolonged.

The observation that reduced glutathione restored the infectivity of some enteroviruses which had been inactivated by PCMB correlates with the previously reported observation of the restoration of hemagglutinating activity by reduced glutathione. The results suggest that sulfhydryl groups are involved in the infection of host cells by these enteroviruses, as well as in the adsorption of enteroviruses to erythrocytes.

Effect of PCMB on the Adsorption of Enteroviruses to Monolayers of Monkey Kidney Cells.—The restoration by reduced glutathione of infectivity which had been inactivated by PCMB made it possible to test whether PCMB prevented adsorption of the virus to the host cell. This possibility was investigated by the following procedure: (*a*) virus suspensions were treated with PCMB; (*b*) monolayer cultures of host cells were inoculated with treated virus suspensions; (*c*) after an adsorption period, the supernatant fluid containing the unadsorbed virus particles was removed and treated with reduced glutathione to reactivate the PCMB-inactivated virus particles.

In such experiments, the inactivation of infectivity by PCMB would be demonstrated by a decreased number of plaques on the original monolayers inoculated in step (*b*). If the inactivation of infectivity was due to prevention of virus adsorption, the inactivated virus would be removed with the supernatant fluid after the adsorption period, and after reactivation with reduced glutathione, the unadsorbed virus should be demonstrable by plaque assay. Experiments of this type were carried with ECHO 7 and polio 2 viruses.

Virus suspensions were diluted 1:100 in TBS or in 0.15 M NaCl buffered at pH 7.2 with 0.01 M tris, and then mixed with an equal volume of PCMB diluted in the same buffer to give a final concentration of 2×10^{-5} M. Controls consisted of virus in the appropriate buffer without PCMB. Mixtures were held at room temperature for 10 minutes, and then diluted in buffer and added to washed monolayers of monkey kidney cells in 10 cm. glass Petri dishes, and the dishes were held at 37°C. for 30 minutes. Buffer, chilled to 4°C., was then added in a volume 4 times greater than that of the inoculum, the monolayers were washed by repeatedly tilting the dishes, and the fluid was removed. The monolayers were then washed twice with 5 ml. of chilled buffer, the overlay added, and the cultures carried through the usual procedure for plaque assay. The number of plaques obtained indicated the amount of infective virus adsorbed to the original monolayers. The supernatant fluids removed from the monolayers were mixed with equal volumes of reduced glutathione diluted to give a final concentration of 5×10^{-3} M. Mixtures were held at room temperature for 30 minutes, and then the infective virus was assayed by the plaque procedure. The plaque counts, corrected for the dilutions inherent in the procedure, indicated the amount of virus which did not adsorb to the original monolayers.

Table IV shows the results obtained in three experiments with ECHO 7 virus. As can be seen in the section dealing with virus which adsorbed to the

original monolayers, there was inactivation of infectivity by PCMB which varied from 86 to 97 per cent. It can also be seen that, after reactivation with reduced glutathione of the supernatant fluids removed from these monolayers, much more infective virus was found in the PCMB-treated samples than in the untreated controls. Thus, a large proportion of the PCMB-treated virus

TABLE IV

Effect of p-Chloromercuribenzoate (PCMB) on the Adsorption of ECHO 7 Virus to Monolayer Cultures of Monkey Kidney Cells

Experiment	Infective virus, PFU			
	Adsorbed to cells		Unadsorbed + unstably adsorbed, removed with washing,*	
	Control	PCMB, 2×10^{-5} M	Control	PCMB 2×10^{-5} M
1	34	3	2	16
2	160	5	12	68
3	110	15	30	140

* Assayed after treatment with reduced glutathione.

TABLE V

Effect of p-Chloromercuribenzoate (PCMB) on the Adsorption of Polio 2 Virus to Monolayer Cultures of Monkey Kidney Cells

Experiment	Infective virus, PFU			
	Adsorbed to cells		Unadsorbed + unstably adsorbed, removed with washing*	
	Control	PCMB, 2×10^{-5} M	Control	PCMB, 2×10^{-5} M
1	36	13	8	11
2	43	7	9	18
3	40	16	10	30

* Assayed after treatment with reduced glutathione.

failed to adsorb to the original cell monolayers. These results indicate that PCMB inactivated the infectivity of ECHO 7 virus particles by preventing their adsorption to monolayers of monkey kidney cells, and that treatment with reduced glutathione restored infectivity.

Table V shows the results obtained in similar experiments with polio 2 virus. In each experiment there was some inactivation of infectivity by PCMB, and after reactivation with reduced glutathione of the supernates removed from cell monolayers, more virus was found in the PCMB-treated samples than

in the untreated controls. Therefore, although the degree of inactivation in these experiments was small, in each experiment there was evidence that PCMB prevented the adsorption of some of the polio 2 virus.

Unstable Adsorption of PCMB-Treated ECHO 7 Virus to Monolayers of Monkey Kidney Cells.—In the above experiments, unadsorbed virus was removed from the monolayers by adding cold buffer and washing the monolayers by repeatedly tilting the dishes. In these experiments the possibility that PCMB-treated virus adsorbed in an unstable manner and was removed by the washing of the monolayers with the cold buffer was not excluded. This

TABLE VI

Unstable Adsorption of p-Chloromercuribenzoate (PCMB)-Treated ECHO 7 Virus to Monolayer Cultures of Monkey Kidney Cells

Diluent	Infective virus, PFU					
	Adsorbed to cells		Unadsorbed, removed without washing*		Unadsorbed + unstably adsorbed, removed with washing*	
	Control	PCMB, 5×10^{-5} M	Control,	PCMB 5×10^{-5} M	Control	PCMB, 5×10^{-5} M
0.15 M NaCl buffered with 0.01 M tris	31	5	9	10	12	27
TBS	26	3	14	24	16	26

* Assayed after treatment with reduced glutathione.

possibility was investigated in experiments in which the supernatant fluids were removed from monolayers without the washing procedure.

ECHO 7 virus suspensions were diluted 1:100 in TBS or in 0.15 M NaCl buffered at pH 7.2 with 0.01 M tris, and then mixed with equal volumes of PCMB solution diluted to give a final concentration of 5×10^{-5} M. Controls consisted of virus in buffer. Mixtures were held at room temperature for 10 minutes, and then diluted in buffer, and added to monkey kidney monolayers. The cultures were held at 37°C. for 1 hour, and then a portion of the supernatant fluid was removed and diluted 1:5 in the appropriate buffer. To the remaining supernatant fluid, cold buffer was added in a volume 5 times that of the inoculum, and the monolayers were washed by tilting the dishes. The supernatant was then removed, and the monolayer was washed again with cold buffer, the overlay added, and the usual plaque assay procedure carried out. The samples of supernatant fluids removed without washing and those removed after washing were treated with reduced glutathione by the above method, and assayed for infective virus by the plaque procedure. In these plaque assays an adsorption period of 1 hour was employed. The plaque counts were corrected for the dilutions inherent in the procedure.

As can be seen in Table VI, when the supernatant fluids were removed from the original monolayers without washing, there was no difference between the control and the PCMB-treated samples in the experiment carried out in

0.15 M NaCl buffered with 0.01 M tris; however, in the experiment carried out in TBS, there was more virus in the supernatant fluid of the PCMB-treated sample than in the control. Thus, in the tris buffer with NaCl as the only salt, PCMB-treated virus apparently did adsorb; but in TBS, which contains Ca++, Mg++, K+, and some phosphate in addition to NaCl, PCMB-treated virus did not adsorb. As also shown in Table VI, when the supernatant fluids were removed from the monolayers with washing, there was more virus in the PCMB-treated samples than in the controls regardless of which buffer was used, a result which is similar to those described above where washing was also carried out. Therefore, although PCMB-treated virus apparently adsorbed in one buffer, it did so in an unstable manner. Thus, PCMB-treated virus did not enter into the same relationship with the cell as the untreated virus, because the PCMB-treated was removed by washing, whereas the untreated virus was not removed.

To investigate further the unstable adsorption of PCMB-treated virus to monolayers of monkey kidney cells, the disappearance of ECHO 7 virus from the supernatant fluid of an inoculum added to monolayers was studied.

Virus suspensions were diluted 1:100 in TBS or in 0.15 M NaCl buffered with 0.01 M tris, and mixed with equal volumes of PCMB solution diluted in the appropriate buffer to give a final concentration of 5×10^{-5} M. Controls consisted of virus in buffer. Mixtures were held at room temperature for 10 minutes, then diluted 1:100 in buffer, and 0.8 ml. volumes were added to glass Petri dishes, 10 cm. in diameter, containing confluent monolayers of monkey kidney cells. Dishes were held at 37°C., and at intervals, 0.1 ml. aliquots were removed, diluted 1:10 in buffer, mixed with an equal volume of 10^{-2} M reduced glutathione and held at room temperature for 30 minutes. The samples were then assayed for infective virus by the plaque technique.

The results of these experiments are shown in Fig. 6. As can be seen in section A, when the experiment was carried out in tris buffer with NaCl as the only added salt there was little difference in the rates of adsorption of PCMB-treated virus and the untreated control virus as measured by the disappearance of virus from the supernatant. This result confirms the above finding that PCMB-treated virus adsorbed, though unstably, when the experiment was carried out in tris buffer with NaCl.

As can be seen in section B of Fig. 6, when the experiment was carried out in TBS, PCMB-treated virus did not disappear from the supernate at the same rate as untreated virus, and after an initial decrease, there was no further change in the concentration of PCMB-treated virus in the supernate. This observation is in agreement with the above finding that PCMB treatment prevented even an unstable adsorption of virus when the experiment was carried out in TBS.

The results of this group of experiments indicate that, depending on the conditions of the experiment, PCMB treatment of ECHO 7 virus either pre-

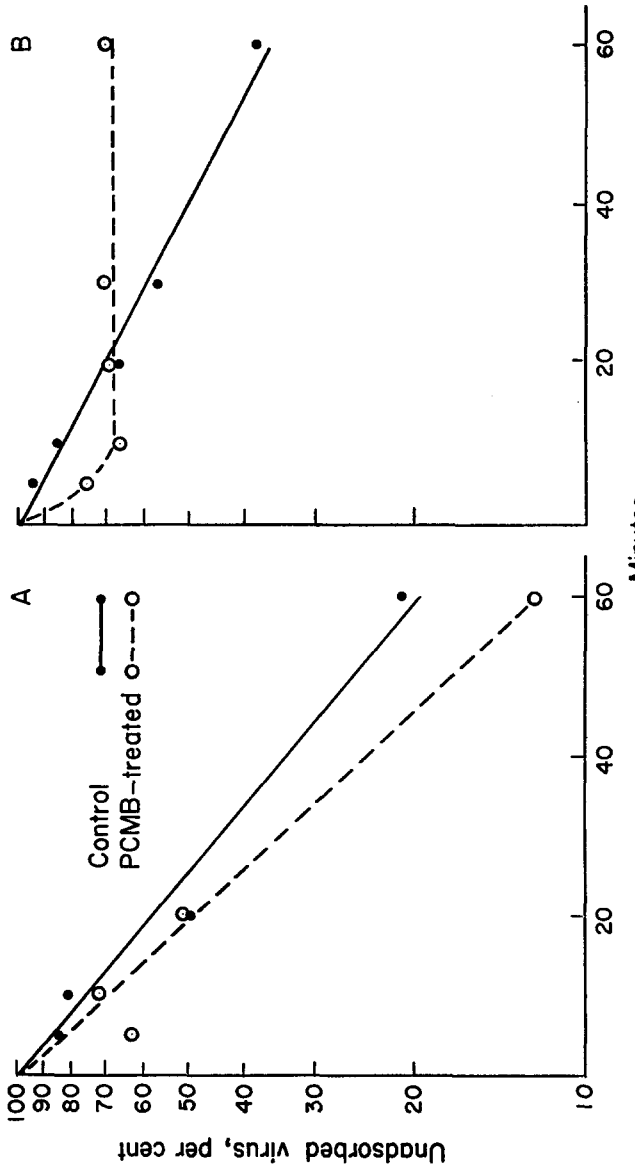

FIG. 6. Effect of *p*-chloromercuribenzoate (PCMB) on adsorption of ECHO 7 virus to monolayers of monkey kidney cells. A. In 0.15 M NaCl buffered with 0.01 M tris(hydroxymethyl)aminomethane (tris), pH 7.2. B. In tris-buffered saline (TBS), pH 7.2, containing NaCl, KCl, $Na_2HPO_4 \cdot 7H_2O$, $MgCl_2$, $CaCl_2 \cdot 2H_2O$, and tris.

vents adsorption to monolayers of monkey kidney cells or results in adsorption which is so unstable that virus is removed by washing.

Effect of PCMB on the Infectivity of Influenza Virus.—It was previously reported that PCMB and mercuric chloride did not prevent agglutination of chicken erythrocytes by influenza virus (1). This indicates that PCMB-treated virus did adsorb to the erythrocytes. The effect of PCMB on the adsorption of influenza virus to the chorioallantoic membrane of the chick embryo was investigated.

Pieces of chorioallantoic membranes of 10 day white Leghorn chick embryos were punched out by the procedure of Tamm (10). Twenty-four pieces, each with an area of 3.8 $cm.^2$, were suspended in 1 ml. of 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate. Allantoic fluid

TABLE VII

Effect of p-Chloromercuribenzoate (PCMB) on the Adsorption of Influenza B Virus to the Chorioallantoic Membrane (CAM) of the Chick Embryo

Treatment of virus	Hemagglutinating units of virus		Virus adsorbed to CAM, per cent of amount originally present
	Before adsorption	Supernate after adsorption	
None	128	<8	>94
PCMB, 1×10^{-4} M	128	<8	>94

infected with the Lee strain of influenza B virus was diluted 1:15 in the above phosphate buffer, and then mixed with an equal volume of PCMB diluted to give a final concentration of 1×10^{-4} M, and incubated for 30 minutes at 37°C. Controls consisted of virus in buffer alone. One ml. volumes of treated mixtures were added to the pieces of chorioallantoic membrane in suspension, and mixtures were held at 4°C. for 1 hour with occasional shaking. The membrane pieces were then sedimented by centrifugation, and the supernate removed and virus assayed in hemagglutination titrations (11).

As can be seen in Table VII, PCMB treatment did not prevent adsorption of influenza virus to the chorioallantoic membrane.

The effect of PCMB on the infectivity of influenza virus was also investigated.

The Lee strain of influenza B and the RI/4$^+$ and RI/4$^-$ substrains (12, 13) of influenza A2 were used. Virus containing allantoic fluid was diluted 1:5 in 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate, and then mixed with an equal volume of 2×10^{-4} M PCMB, or phosphate buffer as a control, and incubated at 37°C. for 15 minutes. Mixtures were then serially diluted in 0.5 log unit steps in phosphate buffer, and infective virus was assayed in 10 day chick embryos as previously described (13).

As can be seen in Table VIII, PCMB caused inactivation of infectivity of each of the three viruses tested. Thus, PCMB inactivates the infectivity of influenza virus by some means other than preventing the adsorption of virus to cells.

The results obtained with influenza virus are in agreement with those of Allison and Valentine (14, 15), who found that PCMB and other sulfhydryl reagents inactivated infectivity of fowl plague virus, but did not affect adsorption to HeLa cell monolayers. They are also in agreement with the earlier studies of Klein and coworkers (16–18), who found that mercuric chloride inactivated influenza virus infectivity, but did not prevent hemagglutination. These authors also suggested that mercuric chloride–treated influenza virus did adsorb to the cells of the mouse lung (17) and chorioallantoic membrane (18) *in vivo*.

TABLE VIII

Effect of p-Chloromercuribenzoate (PCMB) on the Infectivity of Influenza Virus

Virus	Infective virus, EID_{50} per ml.		Fold reduction in EID_{50}
	Control	PCMB 1×10^{-4} M	
Lee	3.1×10^8	1.5×10^7	21
RI/4$^+$	8.3×10^7	8.0×10^6	10
RI/4$^-$	4.2×10^8	1.8×10^7	23

DISCUSSION

Recent extensive studies by McLaren, Holland, and Syverton have shown that cells that are susceptible to infection by enteroviruses possess receptors for these viruses which insusceptible cells do not possess (19–23). Their findings suggest that the receptors are lipoprotein structures (20). There is also evidence which suggests that the receptors on human erythrocytes for hemagglutinating enteroviruses are at least in part protein in nature (24), and may be lipoprotein (25). However, the precise mechanism of adsorption of enteroviruses to cell receptors is unknown.

In a previous communication (1) the inactivation of the hemagglutinating activity of enteroviruses by several sulfhydryl reagents was reported; the inactivating effect of PCMB has been confirmed (26). Evidence has been presented that virus sulfhydryl groups are involved in the adsorption of hemagglutinating enteroviruses to erythrocytes (1). The results obtained in preliminary experiments with several sulfhydryl reagents (1), and in the present detailed experiments with PCMB, indicate that virus sulfhydryl groups are involved also in the process of infection of susceptible cells by enteroviruses. The reversal by reduced glutathione, a thiol compound, of inactivation of infectivity by PCMB made possible the demonstration that PCMB caused inactivation of infectivity by preventing the adsorption of ECHO 7 virus to monkey kidney cell monolayers. These results suggest that sulfhydryl groups

are involved in the adsorption of some enteroviruses to monkey kidney cells in monolayer cultures.

The way in which sulfhydryl groups may be involved in the adsorption of enteroviruses to monkey kidney cells or erythrocytes is at present unknown. Experiments on the adsorption of virus to erythrocytes suggested that disulfide formation between virus sulfhydryl groups and sulfhydryl groups of the cell is unlikely (1); however, a virus sulfhydryl group might react with a disulfide group of the cell in some form of sulfhydryl-disulfide interchange (27).

It should be emphasized that the conditions of the experiments markedly affect the rate and extent of inactivation of enterovirus infectivity by PCMB. The purity of the virus preparation, the buffer used, and the pH (1) all affect inactivation. These findings are compatible with a reaction between PCMB and virus sulfhydryl groups (1, 7, 8, 28).

It should also be emphasized that the degree of reactivation of infectivity by reduced glutathione after inactivation by PCMB depends to a certain extent on the conditions of inactivation. With high concentrations of PCMB, prolonged treatment at 37°C., or when phosphate buffer was used, consistent reactivation of infectivity of ECHO 7 virus could not be obtained. The explanation for this phenomenon is unknown.

Evidence presented in this communication indicates that under certain conditions PCMB-treated enterovirus may adsorb to monolayers of monkey kidney cells, but this adsorption is unstable and does not result in the same virus-cell relationship that occurs with untreated virus. However, under other conditions even such unstable adsorption is prevented by PCMB. Preliminary experiments (29) have suggested that PCMB may not prevent the adsorption of enteroviruses to HeLa or KB cells in suspension. The effect of PCMB on the adsorption of enteroviruses to susceptible cells may also vary with different viruses. The findings with influenza virus reported previously (1) and in this communication, and those reported by others (14–18), indicate that sulfhydryl reagents which are capable of inactivating the infectivity of certain enteroviruses by preventing stable adsorption to monkey kidney cells, can also inactivate the infectivity of myxoviruses without preventing adsorption.

SUMMARY

The infectivity of several enteroviruses was inactivated by the sulfhydryl reagent *p*-chloromercuribenzoate (PCMB). The rate of inactivation was dependent on the ionic environment in which the reaction was carried out. Inactivation of infectivity was reversed by the thiol compound, reduced glutathione. Under certain conditions, PCMB prevented the adsorption of some enteroviruses to monolayer cultures of monkey kidney cells. The results suggest that enterovirus sulfhydryl groups are involved in the establishment of infection, and that they play a role in the adsorption of virus to host cells.

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