Interaction of \textit{p-}Chloromercuribenzoate with Adenoviruses. 
\textit{Inactivation of Haemagglutinins and Degradation of}
\textit{Virions of Types 3, 4 and 7}

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\textbf{SUMMARY}

Interaction of \textit{p-}chloromercuribenzoate with the haemagglutinins of adenovirus types 3, 4 and 7 resulted in the loss of their ability to agglutinate red cells—a loss which can be either reversible or irreversible. The structural integrity of the haemagglutinins of types 3 and 7 was maintained in the process of their reversible inactivation in 0.2 M-tris buffer, but inactivation was irreversible if they were allowed to react with \textit{p-}chloromercuribenzoate in 0.1 M-borate buffer (pH 8.0) or if the reversibly inactivated haemagglutinins were transferred into the borate buffer even in the absence of the mercurial compound. Thus the reversibly inactivated haemagglutinins are predisposed to irreversible loss of activity. Haemagglutinin of type 4 was irreversibly inactivated in both buffers. Virus particles of adenovirus types 3, 4 and 7 are degraded by treatment with \textit{p-}chloromercuribenzoate in the borate buffer.

\textbf{INTRODUCTION}

In recent years several reports have appeared in which the role of sulphhydryl (SH) groups for the maintenance of conformational stability of proteins and preservation of their three-dimensional structure has been demonstrated. The SH group reagent \textit{p-}chloromercuribenzoate splits several proteins such as phosphorylase (3.1.3.17) (Madsen & Cori, 1956), formyl-tetrahydrofolate synthetase (6.3.4.3.) Himes & Rabinowitz, 1962), haemerythrin (Keresztes-Nagy & Klotz, 1963), aspartate transcarbamylase (2.1.3.2) (Gerhart & Schachman, 1965), phycoerythrin (Pecchi & Fujimori, 1967) and haemoglobin (Rosemeyer & Huehns, 1967) into subunits. The same reagent also reversibly dissociates \textit{Escherichia coli} ribosome dimers into monomers (Wang & Matheson, 1967) and the latter particles into subunits (Tamaoki & Miyazawa, 1967). In addition, SH group reagents, since they degrade potato virus X (Reichmann & Hatt, 1961), turnip yellow mosaic virus (Kaper & Houwing, 1962), poliovirus (Philipson, 1964) and foot-and-mouth disease virus (Cowan, 1966) have been tools for study of the macromolecular organization of viruses. The inactivation of the infectivity of several viruses, including adenoviruses, by SH reagents has been described (Allison, Buckland & Andrewes, 1962). The haemagglutinating activity of adenovirus type 7, but not that of type 9 or simian adenovirus type 17, is lost after treatment with \textit{p-}chloromercuribenzoate (Buckland, 1960).
The present communication reports the results of continuing studies on the interaction of SH groups reagents with haemagglutinins and virus particles of adenovirus types 3, 4 and 7.

METHODS

Viruses. Adenovirus type 3 was obtained from Great Lakes Naval Training Station as a throat washing and was passaged three times in human embryonic kidney (HEK) cells and twelve times in human diploid wi-38 cells. Adenovirus type 4 (designated strain CL68578) was obtained from Dr Chanock of the National Institutes of Health, Bethesda, Maryland, U.S.A., after its seventh passage in wi-38 cells. Adenovirus type 7 was obtained from the National Institutes of Health as strain 55142. It had undergone seven passages in HEK cells and eleven passages in wi-38 cells. All three viruses were propagated in human diploid wi-38 monolayer cell cultures in 1000 ml. Blake bottles. The inoculum consisted of $10^{6.7}$ to $10^{7.7}$ TCD$_{50}$ of each virus (the titre obtained on HEK cells). During multiplication of the viruses the cells were maintained in Eagle's medium with 2% agammaglobulin calf serum. The virus material was harvested 6 to 8 days after inoculation. The pool of cells and culture medium was frozen and thawed, clarified by low-speed centrifugation, and concentrated about 50 times by ultrafiltration. The resulting material had an infectivity titre of about $10^6$ TCD$_{50}$/ml. (on HEK cells), a haemagglutination titre of 256 to 1024, and a complement-fixation titre of 128 to 1024 as determined by use of homotypic adenovirus antisera. It was stored frozen at $-20^\circ$ until used. In some experiments adenovirus types 7a and 5 propagated in HeLa cell monolayer cultures were used.

Virus particles from the tissue culture material were concentrated and partially purified by centrifugation at 30,000 rev./min. for 1 hr using a no. 50 rotor and an L-4 preparative ultracentrifuge (Spinco Division, Beckman Instruments, Inc., Palo Alto, California, U.S.A.). The sediments were resuspended in appropriate buffer solutions. The supernatant fluids were used for partial purification of haemagglutinins by centrifuging 2.5 ml. of the fluid layered over 2 ml. of a 12.5% (w/v) solution of sucrose in phosphate-buffered saline (PBS; 0.075 M-phosphate, 0.072 M-NaCl, pH 7.2) for 2 hr at 46,000 rev./min., using the SW 65 rotor. The sediments and the bottom quarter of the sucrose solution were pooled and used for further studies.

Rate zonal centrifugation. Rate zonal centrifugation was performed with linear sucrose gradients (10 to 25% sucrose in PBS) of a total volume of 4 ml. Samples of 0.2 to 0.4 ml. were layered over the gradients and centrifuged at 37,500 rev./min. for 2 hr (for haemagglutinins) or at 18,000 rev./min. for 30 min. (for virus particles), using the SW 65 rotor. Consecutively numbered fractions of 7 drops were collected from the bottom of the tubes.

Gel filtration. For gel filtration experiments, K 25/45 columns filled with Sephadex G-200 (both products of Pharmacia, Uppsala, Sweden) were used. Samples of 3 ml. each were applied on the top of the gel, which had been pre-equilibrated with the elution buffer, 0.45% NaCl, 0.01 M-tris, pH 7.0. Fractions of 5 ml. each were collected by an automatic fraction collector.

Serological techniques. For complement-fixation (CF) tests the microtechnique described by Sever (1962) was used. Rabbit antisera against adenovirus types 2, 3, 4 and 11 were obtained either from the National Institutes of Health, Bethesda, Maryland, U.S.A., or from Microbiological Associates, Inc., Bethesda, Maryland, U.S.A.
Immunizing antigens used in the production of these antisera were prepared in KB tissue cultures and were not purified before immunization. For studies with adenovirus type 7, human convalescent serum was kindly supplied by Dr Chanock.

The quantitative fluorescent precipitin test and the preparation of fluorescein isothiocyanate-labelled antibody preparations were described in previous reports (Neurath, 1965; Neurath, Rubin & Vernon, 1966).

Haemagglutinin titrations were performed as described by others (Norrby, 1966a; Norrby & Wadell, 1967).

**Inactivation of adenovirus haemagglutinins and degradation of virus particles.** The time course of irreversible inactivation of adenovirus haemagglutinins was investigated under the following conditions (if not stated otherwise). To a solution of haemagglutinin, prepared as described above and suspended in 0.1 M-borate buffer (pH 8.0) prewarmed to 37°, p-chloromercuribenzoate (sodium salt, Calbiochem, Los Angeles, California, U.S.A.) was added to a final concentration of 0.01 M. At regular intervals samples of 0.1 ml. were withdrawn into 0.9 ml. of a 0.1% solution of mercaptoethanol in PBS. Haemagglutinins were then titrated using the same solvent as diluent.

Inactivation by N-ethylmaleimide (Calbiochem) was performed under the same conditions, except that p-chloromercuribenzoate was replaced by N-ethylmaleimide.

In other experiments, the interaction between p-chloromercuribenzoate and haemagglutinins or virus particles was allowed to proceed in 0.2 M-tris buffer (pH 7.0) for 1 hr at 37°. The reaction was stopped by addition of cysteine hydrochloride to a final concentration of 0.015 M.

**RESULTS**

**Reversible inactivation of adenovirus type 7 and type 3 haemagglutinins**

In preliminary experiments, the haemagglutinins of adenovirus types 7 and 7a in crude tissue culture fluid were inactivated by treatment with p-chloromercuribenzoate in 0.2 M-tris for 1 hr at 37°, but the addition of cysteine hydrochloride or mercaptoethanol reactivated from 25 to 100% of them. Such reactivation might have been due either to simple removal of p-chloromercuribenzoate from the sites to which it had attached during the course of inactivation or to the reassociation of subunits formed during a hypothetical degradation of haemagglutinin, in a manner similar to the reversible dissociation of some enzymes induced by p-chloromercuribenzoate (Madsen & Cori, 1956; Gerhart & Schachman, 1965) and ribosomes (Tamaoki & Miyazawa, 1967). Further experiments were performed to elucidate this problem.

Haemagglutinin from crude tissue culture material was partially purified by gel filtration on Sephadex G-200 which separates the bulk of serum proteins and virus-specific complement-fixing antigens from haemagglutinin (Fig. 1, top). The fractions containing haemagglutinin were pooled and concentrated by pervaporation to 30 ml. The material was then rechromatographed on Sephadex G-200, half first being treated with p-chloromercuribenzoate in 0.2 M-tris. Haemagglutinin was recovered from the treated material in the same fractions as from the untreated material (Fig. 1), the only difference being that 0.1% mercaptoethanol in PBS was required as diluent for the haemagglutinin titration of treated material. With PBS alone no haemagglutinin could be demonstrated in the same fractions. A control experiment showed that the use of mercaptoethanol did not increase the titre of untreated haemagglutinin prepara-
Fig. 1. Gel filtration on Sephadex G-200 of crude adenovirus type 7 tissue culture material (top) and of haemagglutinin prepurified by gel filtration before (middle) and after (bottom) reaction with p-chloromercuribenzoate. Haemagglutinin; CF antigen. Arrow indicates the fraction in which serum albumin was eluted.
Fig. 2. Rate zonal centrifugation of haemagglutinins of adenovirus types 7 (top), 3 (middle), and 4 (bottom), before (broken line) and after (solid line) treatment with p-chloromercuribenzoate. Activity recovered without addition of mercaptoethanol; activity recovered after addition of mercaptoethanol. Fraction 1 = bottom fraction. Untreated and treated haemagglutinins were centrifuged separately and the results were superimposed on the figure.
tions. This suggests that haemagglutinin can be inactivated by p-chloromercuribenzoate without being degraded into subunits and that the reactivation can be ascribed to the removal of the agent from the sites of its attachment to haemagglutinin by subsequent treatment with mercaptoethanol.

Rate zonal centrifugation experiments with adenovirus type 7 and 7a* haemagglutinins untreated and treated with p-chloromercuribenzoate led to the same conclusion. The peak of haemagglutination activity was recovered in the same fractions with both untreated and treated haemagglutinin (Fig. 2, top). Similar experiments were also performed with the haemagglutinins of adenovirus types 3 and 4. With the former, some reactivation was also observed (Fig. 2, middle). On the other hand, type 4 haemagglutinin was found in separate experiments to be much more sensitive to p-chloromercuribenzoate, and with this type the inactivation time was shortened to 5 min. as compared with 60 min. for the other two. No reactivation was observed, since the addition of mercaptoethanol did not increase the haemagglutination titre of residual haemagglutinins (Fig. 2, bottom). The rate zonal centrifugation experiments also showed that adenovirus type 7 haemagglutinins sedimented at about the same rate as those of types 3 and 4, the properties of which have been already described (Norrby, 1966a; Norrby & Wadell, 1967). The sedimentation coefficients ($S_{w, 20}$), calculated according to the method of Martin & Ames (1960), were all in the range of 55 to 65 S. With type 4 haemagglutinin some haemagglutination activity could be recovered in the top fractions of the gradient. This probably corresponds to incomplete haemagglutinin (Norrby & Wadell, 1967).

Irreversible inactivation of adenovirus type 3, 4, 5 and 7 haemagglutinins by p-chloromercuribenzoate

We consider the inactivation described above as reversible. Irreversible inactivation results in a loss of haemagglutinating activity even when mercaptoethanol is used in the diluent for haemagglutination titrations. The kinetics of irreversible inactivation of adenovirus haemagglutinins by p-chloromercuribenzoate in borate buffer (pH 8.0) differ markedly with different types, the haemagglutinating activity of type 4 being most rapidly destroyed. The results with type 5 agglutinin, included here for purposes of comparison, show it to be the most resistant to the inactivating effect of p-chloromercuribenzoate (Fig. 3 to 5).

Since adenovirus haemagglutinins (at least those of types 3 and 7) could be inactivated both reversibly and irreversibly, further experiments were performed to elucidate the basis of the difference between the two types of inactivation. Haemagglutinins of adenovirus types 3 and 7 were reversibly inactivated by p-chloromercuribenzoate in 0.2 M-tris and subsequently centrifuged under conditions for purification of haemagglutinins. The preparations thus obtained were transferred into a tenfold volume of 0.1 M-borate buffer (pH 8.0) prewarmed to 37° and kept at this temperature. At regular intervals, samples of 0.1 ml. were withdrawn into 1 ml. of 0.1% mercaptoethanol in PBS and the haemagglutinins titrated. Reversibly inactivated haemagglutinins were irreversibly destroyed when transferred to an environment less favourable for their survival (Fig. 3, 4). Since no additional p-chloromercuribenzoate could have attached to haemagglutinins during this process, the irreversible inactivation can be ascribed

* Identical results were obtained with type 7a, but were not included in this report.
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Fig. 3. Irreversible inactivation of adenovirus type 7 haemagglutinin by $p$-chloromercuribenzoate. ○, Inactivation in borate buffer, pH 8·0; ○, inactivation by transfer into borate buffer, pH 8·0, of haemagglutinin pretreated by $p$-chloromercuribenzoate in 0·2 M-tris buffer, pH 7·0; △, inactivation in 0·5 M-histidine buffer, pH 8·0; ■, inactivation in borate buffer, pH 8·0, when the haemagglutination was titrated in the presence of heterotypic adenovirus type 2 antiserum.

Fig. 4. Irreversible inactivation of adenovirus type 3 haemagglutinin by $p$-chloromercuribenzoate. Symbols are the same as for Fig. 3. Heterotypic adenovirus type 11 antiserum was used to test for the presence of incomplete haemagglutinin.
to conformational changes occurring in the \textit{p}–\textit{chloromercuribenzoate} + haemagglutinin complex. In this respect it is noteworthy that the overall rate of inactivation was similar for both direct inactivation in borate buffer (pH 8.0) and inactivation by transfer experiments. Haemagglutinins not pre-treated by \textit{p}–\textit{chloromercuribenzoate} were not inactivated by transfer into this buffer. Similar experiments with adenovirus type 4 haemagglutinin were not made due to its irreversible inactivation under conditions leading only to reversible inactivation with haemagglutinins of types 3 and 7.

![Fig. 5. Irreversible inactivation of adenovirus type 4 haemagglutinin by \textit{p}–\textit{chloromercuribenzoate}. Symbols are the same as for Fig. 3. Results with inactivation of adenovirus type 5 haemagglutinin are included (\textbullet). Heterotypic adenovirus type 11 antiserum was used to test for the presence of incomplete haemagglutinin.](image)

The next point to be considered was the character of the conformational changes resulting in the irreversible inactivation. The results of several experiments suggested that the haemagglutinin of type 4 adenovirus was degraded in the process of inactivation. The distribution of complement-fixing antigen was followed in fractions of original and inactivated type 4 haemagglutinins submitted to rate zonal centrifugations. No complement-fixing antigen could be recovered in those fractions that corresponded to the inactivated material—fractions that would have contained particles of the size of haemagglutinin if such particles had been present. Unlike the case with untreated material, complement-fixing antigen could be recovered only in the top fractions, indicating a breakdown of haemagglutinin into smaller units (Fig. 6). Similar experiments with haemagglutinins of types 3 and 7 could not be made, since they did not fix complement with the antisera used. These experiments indicated the possibility that haemagglutinins were degraded as the result of their reaction with \textit{p}–\textit{chloromercuribenzoate}. Since it had been shown elsewhere (Norrby, 1966b; Norrby & Wadell, 1967) that complete haemagglutinins can be degraded into subunits (incomplete haemagglutinins) by heat treatment, further experiments were needed to determine whether or not the irreversible inactivation might be ascribed merely to such a conversion. If it could, no inactivation of haemagglutinating activity by \textit{p}–\textit{chloromercuribenzoate} should be observed when suitable heterotypic adenovirus antisera were used.
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in the diluent for haemagglutination titrations. The results of the corresponding experiments (Fig 3, to 5) showed that haemagglutinating activity was destroyed even under these conditions of titration. The inactivation therefore could not be attributed to the conversion of complete into incomplete haemagglutinins.

Fig. 6. Degradation of adenovirus type 4 haemagglutinin by p-chloromercuribenzoate. Distribution of haemagglutinin (■) and CF antigen (□) in fractions obtained after rate zonal centrifugation of haemagglutinin before (bottom) and after (top) treatment by p-chloromercuribenzoate, in borate buffer, pH 8.0. Fraction 1 = bottom fraction.

p-Chloromercuribenzoate is not an entirely specific reagent for thiol groups because it also reacts with histidyl residues. A distinction between the latter and SH groups might be made if the inactivation were reversed by histidine (Barnard & Stein, 1958). But in the present work, the inactivation of haemagglutinins was not reversed in a buffer containing 0.5 M-histidine hydrochloride adjusted by NaOH to pH 8.0, i.e. under the conditions of a 50-fold molar excess of histidine over p-chloromercuribenzoate (Fig. 3 to 5). These results suggest that p-chloromercuribenzoate interacts exclusively with the SH groups of the haemagglutinins.
Irreversible inactivation of adenovirus types 3, 4, 5 and 7 haemagglutinins by N-ethylmaleimide

To confirm the key role of the blocking of SH groups in the inactivation of haemagglutinins, another SH group reagent, N-ethylmaleimide, was tested (Fig. 7). This reagent also inactivated the haemagglutinins, though relatively more slowly than p-chloromercuribenzoate. Although N-ethylmaleimide has been widely used as a specific thiol group reagent, it may react with functional groups in protein other than thiols—namely with imidazole, e-amino and terminal α-amino groups (Brewer & Riehm, 1967). Therefore, inactivation experiments with N-ethylmaleimide were also performed in 0.5 M-histidine hydrochloride under conditions similar to those used with p-chloromercuribenzoate. No evidence of decreased rate of inactivation could be found. For clarity, the corresponding results were omitted from Fig. 7.

Degradation of adenovirus types 3, 4 and 7 virus particles

Particles of adenovirus types 3, 4, and 7 were treated with p-chloromercuribenzoate under conditions leading to irreversible inactivation of the corresponding haemagglutinins and were subsequently submitted to rate zonal centrifugation. No visible virus bands were observed, compared with the bands in untreated material, and no or very low levels of complement-fixing and precipitating antigens were recovered from the gradient fractions in which intact particles, if present, should have been detectable (Fig. 8 to 10). Instead, increased levels of complement-fixing and precipitating antigen were found in the top fractions from density gradients of virus material treated with p-chloromercuribenzoate, indicating the degradation of particles. The occurrence
of some complement-fixing and precipitating antigen in the top fractions of the untreated virus material may be ascribed to partial damage to particles during storage and/or to contamination with soluble antigens incompletely removed from the particles in the process of their partial purification.

Fig. 8. Degradation of type 3 adenovirus particles by p-chloromercuribenzoate. Distribution of precipitating antigen in fractions obtained after rate zonal centrifugation of virus preparations before (bottom) and after (top) treatment by p-chloromercuribenzoate in borate buffer, pH 8.0. Fraction 1 = bottom fraction.

When treated with p-chloromercuribenzoate in 0.2 M-tris, type 4 virus particles were degraded while type 7 were not. Thus the degradation of virus particles by p-chloromercuribenzoate was correlated with the susceptibility of the haemagglutinins to irreversible inactivation by this agent.
DISCUSSION

The studies on the interaction of adenovirus haemagglutinins with p-chloromercuribenzoate indicate the formation, in the course of the reaction, of a p-chloromercuribenzoate + haemagglutinin complex in which the integrity of haemagglutinin is preserved but the ability to agglutinate red cells is lost. This loss of haemagglutinating activity is reversible and can be restored by the addition of thiols, as shown with haemagglutinins of types 3 and 7, and may be due either to the blocking by p-chloromercuribenzoate of sites involved directly in the adsorption of haemagglutinin to red
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cells, or to the steric hindrance caused by the binding of \( p \)-chloromercuribenzoate to sites adjacent to those responsible for haemagglutination, or to conformational changes in haemagglutinin caused by the binding of \( p \)-chloromercuribenzoate. If the \( p \)-chloromercuribenzoate + haemagglutinin complex is transferred to a less favourable environment or if the interaction of \( p \)-chloromercuribenzoate with haemagglutinin is allowed to proceed in such an environment, the haemagglutinating activity can no longer be restored by the addition of thiols. Whether or not the degradation of

**Fig. 10.** Degradation of type 7 adenovirus particles by \( p \)-chloromercuribenzoate. Conditions of treatment and explanations are the same as for Fig. 9.

haemagglutinins is a prerequisite for their irreversible inactivation was not determined. The degradation of haemagglutinins per se, however (as demonstrated here for type 4) is probably not responsible for the loss of haemagglutinating activity, since this activity could not be restored by the addition of heterotypic adenovirus antisera. Therefore, treatment by \( p \)-chloromercuribenzoate either does not convert complete haemagglutinins into incomplete ones (Norrby, 1966b; Norrby & Wadell, 1967) or the latter are formed but inactivated in a subsequent step of the interaction. Under conditions of treatment with \( p \)-chloromercuribenzoate leading to irreversible inactivation of haemagglutinins, similar treatment of virus particles leads to their degradation. It is
highly probable that the SH groups of virus particles (haemagglutinins) are responsible for their interaction with p-chloromercuribenzoate and N-ethylmaleimide. To clarify this point, further studies should compare the actual number of molecules of these reagents bound per particle with the number of SH groups blocked. It would also be interesting to learn how this number would compare with the number of half-cystine residues, several thousands of which could be expected per virus according to data on the amino acid composition of adenoviruses (Polasa & Green, 1967).

The infectivity of adenoviruses can be irreversibly inactivated by p-chloromercuribenzoate under conditions in which virus particles maintain their structural integrity (unpublished data). A similar phenomenon observed with turnip yellow mosaic virus has been attributed to the degradation of ribonucleic acid inside the intact protein capsid (Kaper & Jenifer, 1965). Whether the same phenomenon is responsible for the loss of infectivity with adenoviruses remains to be determined.

In the present effort to demonstrate the effect of SH group reagents on adenoviruses, we have chosen rather arbitrarily the conditions for inactivation and have used serological techniques and haemagglutination titrations in conjunction with rate zonal centrifugation. To characterize the interaction between p-chloromercuribenzoate and adenovirus particles and other ‘soluble’ viral components formed in the course of infection of cells and to elucidate the individual steps occurring during this interaction, more detailed biochemical studies analogous to those performed with turnip yellow mosaic virus (Kaper & Houwing, 1962a, b; Kaper & Jenifer, 1965, 1967; Godschalk & Veldstra, 1965) would be required. The greater complexity of the adenoviruses and their virus-specific ‘soluble’ components, as well as their differences in susceptibility to inactivation by p-chloromercuribenzoate, should make such studies intriguing.

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