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Studies on the Red Cell and Antibody-Reactive Sites of the Parvovirus H-1: Effect of Fixatives[1] (37891)

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Previous studies have shown that hemagglutination (HA) of the H-viruses, members of the Parvovirus group, is individually specific and diagnostic with respect to the types of erythrocytes agglutinated (1); is resistant to proteolytic enzyme attack, and, in fact, is enhanced by papain (2) and ficin (unpublished data); is resistant to a wide range of pH and heat (3), and, in the case of two of the H-viruses, namely, H-1 and HB, is inhibited by a glycoprotein fraction of human placental fluid (4). In addition, the reversible sensitivity of the HA to *p*-chloromercuribenzoate (PCMB) indicates that virus-bound sulfhydryl (SH) groups may be important to the H-1-erythrocyte union (2, 5).

Studies underway in our laboratory on the immunoelectron microscopy of H-1 infected hamster embryo (HE) cells required that the effect of tissue fixatives on the HA and antigenic properties of purified H-1 preparations be determined. The fixatives examined were glutaraldehyde and formaldehyde, both of which react with and crosslink polypeptides. Glutaraldehyde prefers amino, imidazole, and sulfhydryl groups of amino acids (6), while formaldehyde crosslinks amino groups by methylene bridging (7). Glutaraldehyde is superior to formaldehyde with respect to fine structure preservation since it forms stable crosslinks more rapidly (7).

The failure to detect H-1 antigens by fluorescent-antibody labeling in glutaraldehyde-fixed infected cells prompted a study on the effect of several concentrations of the fixatives on the HA activity, antigenicity, and infectivity of purified H-1 preparations. Since our findings appear pertinent to immunomicroscopy in general, and to clarification of the nature of the red cell and antibody binding sites on H-1 virions in particular, results of these studies are reported herein.

Materials and Methods. Virus. Cell culture and virus propagation methods have been described elsewhere (8). Virus was purified by a detergent method, employing homogenization of sodium dodecyl sulfate (SDS)-disrupted cells, sucrose velocity sedimentation, and CsCl isopycnic centrifugation (9). Since it has been found that virus yields from 7–8 H-1 virus-injected newborn hamsters generally exceeds that of a typical tissue culture isolate (unpublished data), in several cases virus was purified from quick-frozen dead or dying newborn hamsters that had been injected subcutaneously with purified H-1 4–5 days previously. An initial 5-min homogenization of the entire animal in buffer (0.05 *M* Tris–HCl, pH 7.4 containing 0.1 *M* NaCl–0.001 *M* EDTA at 5°), followed by a shorter disruption (1.5 min) in 1% SDS–buffer at 26° preceded the steps outlined above. Dilutions of all concentrated virus preparations were made until the HA titer of starting solutions was between 1280 and 5120 units. Virus and reagent dilutions were done with phosphate-buffered saline (PBS)–(0.02 *M* PO_4–0.13 *M* NaCl, pH 7.2).

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Treatment of virus with reagents. HA assay. Purified H-1 in distilled H_2O (0.5 ml) was mixed with an equal volume of 2X PBS (control) or 2X solutions of fixatives in 2X PBS to the final desired concentration. Unless the effect of temperature on the reaction was being studied, all solutions were kept cold (5°) and the virus–reactant mixture incubated immediately for the desired time at 5°. Reactions were stopped by diluting the mixtures to 9 ml with cold PBS and centrifuging the solutions at 40,000 rpm for 3 hr. The resulting virus pellets were then resuspended to 1 ml with cold PBS for the specified assays or to 200 μliters with 0.1 *M* PO_4, pH 7.2, for agar gel immunodiffusion or to 200 μliters with 1% SDS–1% mercaptoethanol (ME) in 0.01 *M* PO_4, pH 7.2 for disruption and subsequent gel electrophoresis. Dialysis was also used to eliminate the fixatives after reaction. The HA assay was done as previously reported (2). HA titers of parallel samples were generally higher for the dialyzed aliquots than for those which had been centrifuged, indicating incomplete sedimentation of virus. The interval between reacting the virus with fixatives and assaying for changes in HA, hemagglutination inhibition (HA-I), or infectivity was kept to a minimum. Infectivity assays showed that both centrifugation and dialysis were equally effective in eliminating unbound fixatives. Glutaraldehyde was obtained from Electron Microscopy Sciences as 10 or 8% solutions in sealed ampules. Formaldehyde was generated from paraformaldehyde by the method Karnovsky (10).

Infectivity assay. Plaque assays of treated and control H-1 preparations were done on single samples previously tested for HA and HA-I, or on parallel aliquots tested separately for HA or HA-I. The method of Ledinko (14) was used except that a line of SV40-transformed human newborn kidney cells (NB) (15) were used for plaque titration in place of Salk "monkey heart" (SMH) cultures. Data are expressed as plaque-forming units (PFU) per ml of treated and untreated virus preparations.

Antigenicity tests. Preparation of gamma globulin. Ouchterlony gel diffusion. Hemagglutination-inhibition (HA-I). Two tests for antigenicity were employed: gel diffusion and HA-I. Anti-H-1 gamma globulin from hamsters immunized with purified H-1 was obtained by fractionating the immune serum on diethylaminoethyl cellulose (DEAE) columns at 5° (11). Samples were eluted with 0.02 *M* PO_4, pH 7.6, and tested against purified H-1 by radial immunodiffusion in Ouchterlony agar (1%) plates (12). Figure 1 shows the immunoprecipitin lines formed when DEAE-purified anti-H-1 gamma globulin is tested against purified H-1 virus. The HA-I tests were performed as described by Clarke and Casals (13), except that kaolin treatment of the purified gamma globulin was not required.

Polyacrylamide gel electrophoresis. Solubilization of treated and control H-1 samples and separation of the polypeptides on SDS-acrylamide gels was done as described in detail elsewhere, except that 10% gels were also used (16).

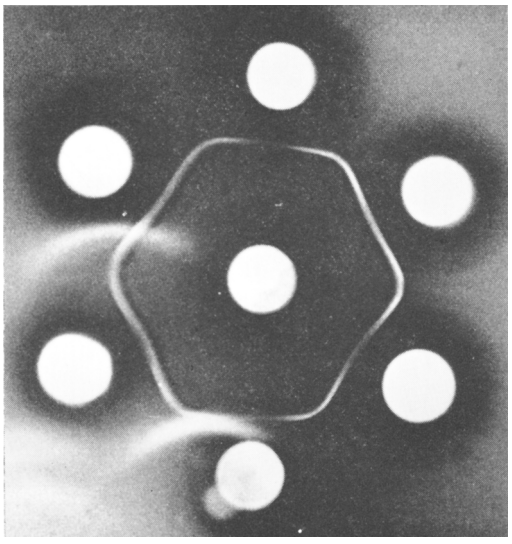

FIG. 1. Agar gel diffusion of Ouchterlony (12). Five microliters of DEAE-purified anti-H-1 gamma globulin was placed in the center well and 5 μliters of purified H-1 in each of the outside wells. Diffusion was allowed to occur for 24 hr at 37° after which immunoprecipitin lines could be seen using incident light. Plates were then flooded with ferritin-conjugated IgG fraction of rabbit anti-hamster gamma globulin (Cappel Laboratories, Inc., Downingtown, PA) and the Prussian blue reaction was done (21).

Results. HA activity. At 5° phosphate-buffered glutaraldehyde in concentrations greater than 1% (0.1 *M*) abolished all HA activity of purified H-1 samples (Table I). This concentration is 25% of that normally used in this laboratory to preserve cellular morphology for electron microscopy and 16% of that known to inhibit the HA of unpurified H-1 (5). The results were the same whether the glutaraldehyde was removed from the virus sample by dialysis, or the virus from the glutaraldehyde solution by centrifugation (Table I).

Formaldehyde, on the other hand, even at 4% (1.33 *M*), had no effect on HA (Table I).

Effect of temperature. When virus and fixatives were reacted at 37°, the HA was abolished at concentrations of fixative that had no effect at 5° (Table II; see also Tables I and III). Increasing the rate of reaction, therefore, quickly produces excessive crosslinks that destroy H-1 HA activity. Glutaraldehyde and formaldehyde added together at 5° produced an additive effect with a resulting reduction in HA and infectivity titer of 93.75 and 99.98%, respectively (Table II).

Effect of fixation on gel electrophoretic mobility. Disruption of treated and control H-1 samples with 1% SDS–1% ME at 100° for 1 min was followed by separation of the polypeptides on 7.5% acrylamide gels. Polypeptides from virions treated with 0.25% glutaraldehyde or 4% formaldehyde had mobilities equal to that of the untreated H-1 control, although the recovery of dissociated polypeptides was less (Fig. 2). Fixation of H-1 with 2.5% glutaraldehyde resulted in poor recovery of virus polypeptides after SDS disruption, indicating that excessive crosslinking of capsid proteins probably occurred, and prevented disaggregation by hot SDS. The polypeptides which were released gave very diffuse bands, suggesting a continuum of different forms of each resulting from intramolecular crosslinking.

The use of high-resolution gels has revealed that, in addition to the previously reported capsid components VP1, VP2, and VP3 (16), the major virion protein VP2 is resolved into two bands, VP2 and VP2′.

TABLE I. Hemagglutination (HA) of Guinea Pig Red Cells by Glutaraldehyde and Formaldehyde-Treated H-1 Virus.[a]

Virus and treatment	Molarity of reagent	HA titer[b] Expt. 1	2	3	4	5	6	7	8[c]	Mean	Infectivity of Expt. 5[d]
H-1 + PBS (control)	—	9	7	6	8	9	9	6	7	7.6	1×10^7 PFU
H-1 + 0.25% glutaraldehyde	0.025	8	6	6	8	9	10	6	8	7.6	1×10^6
H-1 + 0.50% glutaraldehyde	0.05	—	—	4	5	6	—	—	—	5.0	1.5×10^6
H-1 + 1.0% glutaraldehyde	0.10	—	—	0	4	1	6	3	0	2.3	1.25×10^5
H-1 + 1.5% glutaraldehyde	0.15	—	—	0	0	0	—	—	—	0	0
H-1 + 2.0% glutaraldehyde	0.20	—	—	0	0	0	—	—	—	0	0
H-1 + 2.5% glutaraldehyde	0.25	0	0	0	0	0	0	—	0	0	0
H-1 +2.7% formaldehyde + 0.2% picric acid	0.90	8	7	—	—	—	—	—	—	7.5	—
H-1 + 4% formaldehyde	1.33	8	7	—	—	—	10	—	8	8.3	—
H-1 + 2.5% formaldehyde + 0.25% glutaraldehyde	0.83 0.025	—	8	—	—	—	—	—	—	8	—

[a] Virus and reagents were incubated for 30 min at 5° and then separated by centrifugation at 40,000 rpm for 3 hr. The pellets were then resuspended to the original volume (1 ml) and tested for HA activity.

[b] Titers are expressed as that dilution at which hemagglutination was complete. 1 = 1:10; 2 = 1:20; 3 = 1:40; 4 = 1:80; 5 = 1:160; 6 = 1:640; 8 = 1:1280, etc.

[c] In this experiment reactants were dialyzed against 500 ml of PBS at 5° overnight after incubation.

[d] Plaque-forming units per ml of treated or untreated H-1 preparations.

TABLE II. Hemagglutination (HA) of Guinea Pig Red Cells by Glutaraldehyde and Formaldehyde-Treated H-1. Effect of Temperature.[a]

Virus and treatment	HA Titer[b]					
	Expt. 1 37°		Expt. 2 37°	Expt. 3		Infectivity of Expt. 3[c] (PFU/ml) 5°
	Dialysis	Cent.	dialysis	37° Cent.	5° Cent.	Cent.
H-1 + PBS (control)	12	10	13	10	9	9.2×10^7
H-1 + 0.25% glutaraldehyde	0	0	0	2	8	9.0×10^6
H-1 + 4.0% formaldehyde	0	0	0	0	8	2.5×10^7
H-1 + 0.25% glutaraldehyde, 4.0% formaldehyde	2	0	0	0	5	2.5×10^4

[a] Virus and reagents were combined for 30 min at 37° or 5° and then separated by centrifugation at 40,000 rpm for 3 hr or by dialysis at 5° against 500 ml of PBS overnight.

[b] Titers are expressed as that dilution at which hemagglutination was complete. See legend for Table I.

[c] Plaque-forming units per ml of treated or untreated H-1 sample.

Despite the fact that virion disaggregation is done in the presence of 1% mercaptoethanol, and even when 0.1 *M* cysteine is present in the electrophoresis buffer, the splitting of the VP2 band occurs, so that this must be due to causes other than that responsible for the similar finding in Sindbis virus (17). The major virion protein of another parvovirus, the densonucleosis (DNV) virus of a lepidopteran, has also been reported to form a double VP2 band in gels of higher concentration (18). The reason for this is currently under investigation.

Infectivity. Glutaraldehyde at 0.25% (0.025 *M*) reduced H-1 infectivity by 90% while the HA activity of a parallel, treated virus sample remained unchanged (Tables I, II, and III). Further increases in glutaraldehyde gradually lowered both the HA and infectivity of H-1 (Table I, expt. 5). The

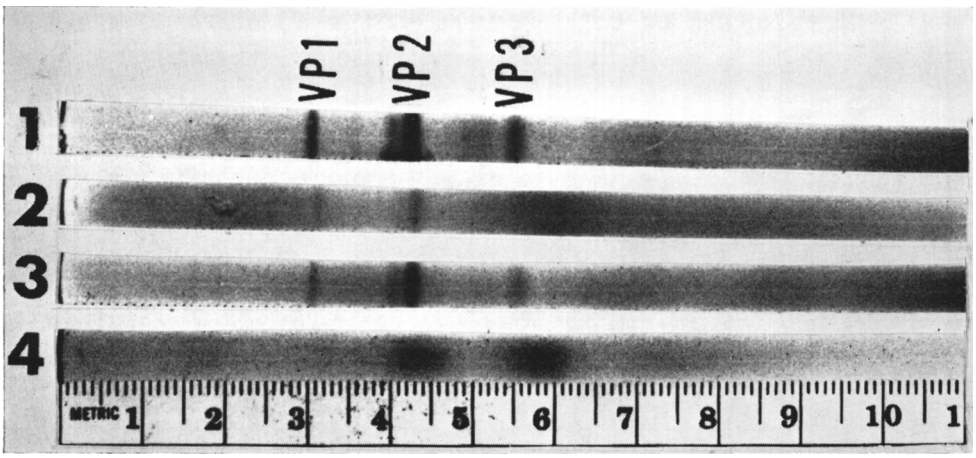

FIG. 2. Capsid proteins of treated and untreated H-1 virions. Virions were disrupted by heat and SDS and separated by electrophoresis on 7.5% gels (16). 1. H-1 + PBS; 2. H-1 + 0.25% glutaraldehyde; 3. H-1 + 4% formaldehyde; 4. H-1 + 2.5% glutaraldehyde. Protein fractions were stained with Coomassie brilliant blue (16). Migration is toward the anode at the right.

infectivity of the same virus sample tested previously for both HA activity and antigenicity (HA-I) was decreased by 90% after treatment with 0.25% glutaraldehyde or 4% formaldehyde (Table III). The combination of glutaraldehyde and formaldehyde was more effective in destroying infectivity than was each compound acting separately (Tables II, III). The drop in infectivity caused by glutaraldehyde and formaldehyde was the same whether dialysis or centrifugation was used to eliminate unbound fixative (Table III). These results imply either a swift penetration of the virions by the fixative to react with the DNA or an irreversible attachment to the capsid proteins preventing subsequent uncoating, since the infectivity rather than the attachment of the virions seems more impaired.

HA-I assay. Glutaraldehyde at 0.25% preserves both H-1 HA activity and antigenicity (Tables I, II, and III). Further increases in the glutaraldehyde concentration to 1% abolished HA activity of H-1, and such preparations, therefore, could not be used for HA-I tests. In an experiment not shown here, exposing H-1 to 0.5% glutaraldehyde for increasing periods (0–60 min) at 5° did not affect its ability to bind subsequently to antibody or red cells. Formaldehyde was also innocuous to both the H-1 virus red cell and antibody-binding sites (Table III).

Immunogel diffusion. Since higher concentrations of glutaraldehyde abolished HA activity of H-1, the question of whether or not antigenicity remained after such treatment could not be answered by the hemagglutination-inhibition assay. Immunogel diffusion, however, as developed by Ouchterlony (12) is a sensitive method for measuring the antigenicity of proteins. Treated and control H-1 samples were tested by resuspending the virus pellets in PBS after centrifugation and allowing them to diffuse in 1% agar against a central well containing purified anti H-1 globulin. Figure 3A and B shows that while 0.25% glutaraldehyde and 4% formaldehyde did not impair the formation of immunoprecipitin lines by treated H-1 and gamma globulin, 2.5% glutaraldehyde apparently destroys the antigenic groupings of H-1.

Discussion. During studies of H-1 virus

TABLE III. Hemagglutination (HA), Hemagglutination-Inhibition (HA-I), and Infectivity Tests of Formaldehyde and Glutaraldehyde-Treated H-1.[a]

Virus and treatment	HA[b]	HA-I[c]	Infectivity[d]
Reagents removed by dialysis			
H-1 + PBS (control)	9	8	3.7×10^8
H-1 + 0.25% glutaraldehyde	10	8	7.5×10^6
H-1 + 4.0% formaldehyde	10	8–9	1.4×10^7
H-1 + 0.25% glutaraldehyde, 4.0% formaldehyde	7	8	4.5×10^4
Reagents removed by centrifugation			
H-1 + PBS (control)	7	8	5×10^7–1×10^8
H-1 + 0.25% glutaraldehyde	6	8	1×10^7
H-1 + 4.0% formaldehyde	7	8	6×10^6
H-1 + 0.25% glutaraldehyde, 4.0% formaldehyde	0	—	1×10^3

[a] Virus and reagents were incubated for 30 min at 5° and then separated by centrifugation at 40,000 rpm for 3 hr or by dialysis at 5° against 500 ml of PBS overnight. The virus pellets resulting from centrifugation were resuspended to the original volume (1 ml) and tested for their ability to bind to red cells, bind anti-H-1 globulins or produce plaque formation on NB cell monolayers.

[b] See legend for Table I.

[c] HA-I titer is taken as the highest dilution of anti-H-1 globulin which causes complete or near complete inhibition of 8 units of H-1 (13).

[d] See legend for Table II.

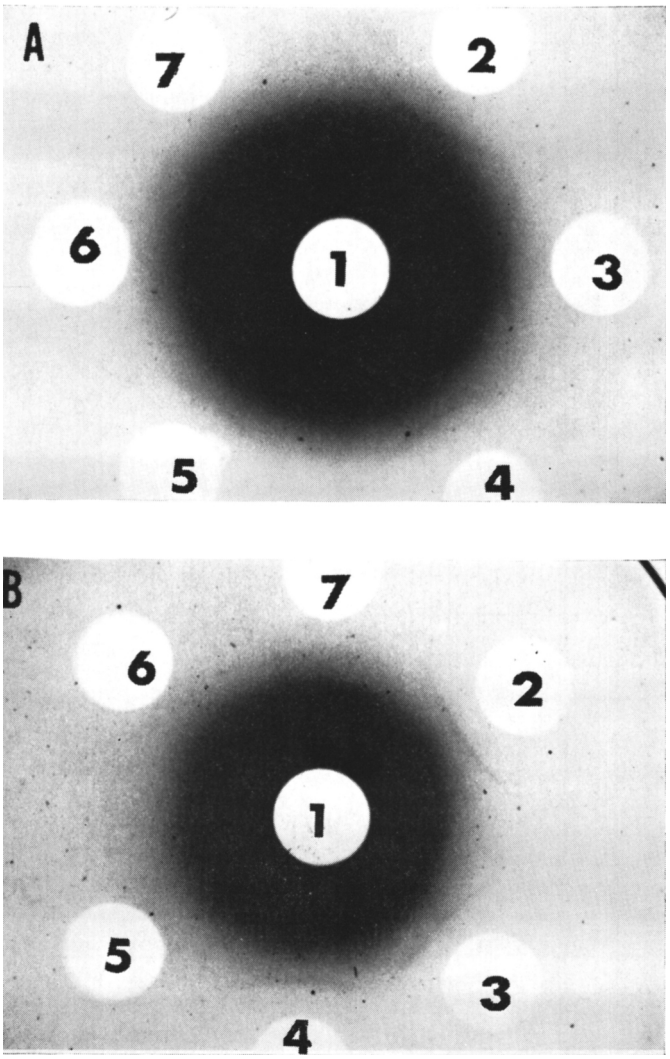

FIG. 3. Ouchterlony gel diffusion of treated and untreated H-1 virions. Center wells (1) contain anti-H-1 gamma globulin. A. Wells 2–4, H-1 + PBS; Wells 5–7, H-1 + 4% formaldehyde. B. Wells 2–4, H-1 + 0.25% glutaraldehyde; Wells 5–7, H-1 + 2.5% glutaraldehyde. Diffusion allowed to proceed for 2 days at 26°. Plates were then washed with 20% methanol in 0.15 *M* NaCl and stained with 0.1% Coomassie blue in 7.5% acetic acid containing 50% methanol.

antigen localization prior to immunoelectron microscopy, it was found that 2.5% glutaraldehyde abolished the reactivity of infected cells to anti-H-1 Fluorescein-labeled antibody. This is apparently not the case with another parvovirus, e.g., the densonucleosis virus of lepidoptera (19). In this study, we examined the effects of various fixatives on H-1 virus.

Concentrations of glutaraldehyde greater than 1% abolished the hemagglutinating activity and infectivity of H-1 preparations. Antigenicity as measured by Ouchterlony precipitin lines was lost, as well as the reactivity of infected cells with cytochrome *c*-labeled anti-H-1 globulin. Formaldehyde, on the other hand, at 4% produced no significant loss of HA, but caused some loss of

infectivity. Considerable antigenicity of the viral proteins remained as seen by the precipitin lines in agar and in the retention of sensitivity to hemagglutination-inhibiting antibodies. Glutaraldehyde (0.25%) caused similar effects to those of 4% formaldehyde, with greater loss of infectivity than HA or antigenicity.

A combination of 4% formaldehyde and 0.25% glutaraldehyde also led to considerable loss of infectivity with variable retention of HA and antigenicity; the loss of infectivity with the combination was much greater, in proportion to the loss of HA, than with either fixative, alone. This greater sensitivity of infectivity than the other parameters can be attributed to the interaction of the fixatives with the single-stranded DNA of the virus (20) and also, possibly to the crosslinking action of the fixatives which would make uncoating of the virus more difficult. This was well substantiated by the polyacrylamide gel electrophoresis studies in which the 2.5% glutaraldehyde fixed virus yielded virtually no capsid proteins after the usual disruption and reduction in the presence of hot SDS and ME.

The nature of the interaction between proteins and the fixatives would preclude their use as fine tools for distinguishing between the identity or nonidentity of the red cell attachment groups and the antigenic sites on the viral capsid. The loss of both parameters seemed simultaneous and the constancy of the HA-I titers implied very similar reactions to the fixatives of the HA site and the antigenic site involved in HA-I, as would be anticipated.

Experiments based on these results, to be detailed elsewhere (I.S. manuscript in preparation), bear out the efficacy of formaldehyde fixation in preserving the antigenicity of H-1 capsid components in infected tissues. However, low concentrations of glutaraldehyde (0.25%), despite the fact that antigenicity, as measured by HA-I and gel diffusion, is retained, also abolish the reactivity of the viral antigens with specific antisera in H-1-infected H.E. monolayers. Glutaraldehyde consistently gives better preservation of the plasma membranes and cytoplasm when compared to formaldehyde. It is possible, therefore, that the superior preservation of the plasma membrane by glutaraldehyde prevents entry of the immunoconjugate into the cell. We plan to examine the application of labeled antibody to ultrathin sections of H-1-infected cells to avoid permeability barriers. We will evaluate further the suitability of formaldehyde and glutaraldehyde as fixatives for immunoelectron microscopy of this system.

Summary. Treatment of the Parvovirus, H-1, with the fixatives formaldehyde and glutaraldehyde have shown that:

1. Formaldehyde, even at 4% (1.33 *M*), had no effect on the red cell or antibody-binding sites of the virus. The infectivity of such samples, however, was abolished, indicating either a direct interaction with the DNA and/or that uncoating of the treated virions is impaired.
2. Glutaraldehyde at 0.25 and 0.5% (0.025 and 0.05 *M*) did not damage the HA or antigenicity, but further increases in bound glutaraldehyde lowered the HA and simultaneously, as determined by immunogel diffusion and SDS-electrophoresis, the ability of the virions to bind anti-H-1 globulin. Glutaraldehyde was as effective as formaldehyde in destroying H-1 infectivity.
3. The loss of HA activity of treated H-1 is dependent upon the temperature of the reaction.
4. Formaldehyde, by virtue of its lack of effect on H-1 HA activity and antigenicity at concentrations permitting reasonable preservation of morphology may be the fixative of choice for immunoelectron microscopy of H-1-infected cells.

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