

# Hemagglutination by Rabies Virus

ERNST KUWERT,<sup>1</sup> TADEUSZ J. WIKTOR, FRANTISEK SOKOL, AND HILARY KOPROWSKI

*The Wistar Institute of Anatomy and Biology and the World Health Organization Reference Centre for Rabies at The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104*

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Goose erythrocytes were agglutinated by five strains of rabies virus grown in monolayer cell cultures at pH 6.4 and at 0 to 4 C. Hemagglutination was not affected by the cell type in which the virus was grown. Prerequisites for occurrence of hemagglutination are absence of hemagglutination inhibitors (such as those contained in bovine serum) and a relatively high virus concentration ( $> 10^6$  plaque-forming units of virus per ml). "Soluble" hemagglutinin was not present in crude preparations of extracellular virus. Treatment of purified preparations of extracellular virus with Tween 80 and ether did not result in release of a "soluble" hemagglutinin. The hemagglutinating property of extracellular virus seemed to be conditioned by the integrity of its coat. Preparations of infectious intracellular virus exhibited about 15 times lower hemagglutinating activity than extracellular virus. This decreased hemagglutinating activity did not seem to be caused by binding of hemagglutination inhibitors to the virus particles. Rabies virus can be quantitatively adsorbed onto and eluted from erythrocytes. Erythrocytes pretreated with rabies virus retained their ability to be agglutinated by the same virus strain. The reaction with rabies virus of erythrocytes treated with the receptor-destroying enzyme or  $KIO_4$  was the same as that of nontreated erythrocytes. The hemagglutinating component of rabies virus, therefore, does not exhibit neuraminidase activity. Treatment of extracellular virus by various agents indicated that the hemagglutinating component consists of protein or lipoprotein. Sulfhydryl groups present in the viral hemagglutinin are essential for hemagglutination.

Until recently, only three methods for the assay of rabies virus were available: titration of infectivity in animals, plaque assay in cell cultures (36), and complement fixation (CF). The latter method (24) measures both infectious virus and all non-infectious viral antigens. In 1968, Halonen et al. (12) reported that rabies virus exhibits hemagglutinating activity (HA). This paper deals with the characterization and mechanism of this phenomenon.

## MATERIALS AND METHODS

**Virus strains.** The Pitman-Moore (PM), Pasteur, and Flury high egg passage (HEP) strains of rabies virus adapted to growth in human diploid cell strain WI-38 (42) were clone-purified three times in agarose-suspended BHK/13S cells (36). The CVS-11 strain of rabies virus propagated in primary hamster kidney cell cultures (22; obtained through the courtesy of P. E. Halonen, National Communicable Disease Center, Atlanta, Ga.) was also purified by cloning three times in BHK/13S cells. The ERA strain of rabies virus (1) was isolated from the attenuated

rabies vaccine lot serial no. 128-2 (Connaught Laboratories, Toronto, Canada) and was propagated for two passages in BHK-21 cell cultures. Seed pools of these virus strains were prepared in BHK-21 cultures.

Parainfluenza virus 1 (Sendai) used in the present study was serially propagated in embryonated eggs. The infectious allantoic fluid contained  $10^{9.7}$  EID<sub>50</sub> and 3,200 hemagglutinating units (HAU) per ml.

**Tissue cultures.** The human diploid cell strain WI-38 (15) and two hamster cell lines, BHK-21 clone 13 (29) and Nil-2 cells (4), were propagated in 1-liter Blake bottles as described by Kaplan et al. (21).

**Infection of cells and animals.** Cell cultures were infected as described previously (39) and incubated at 33 C for 72 hr in the presence of Eagle's basal medium (BME) supplemented with bovine serum albumin (12, 13). Mice and hamsters were inoculated intracerebrally with 1,000 LD<sub>50</sub> of virus. Brains were harvested 5 days after infection.

**Titration of virus infectivity.** The infectivity of tissue culture-adapted strains was determined by a plaque assay technique in agarose-suspended BHK/13S cells as described previously (36; T. J. Wiktor et al., *J. Immunol.*, *in press*) and was expressed as plaque-forming units (PFU) per ml.

Virus contained in brain suspension was titrated in mice. Serial 10-fold dilutions of the virus were

<sup>1</sup> Permanent address: Virologische Abteilung, Institut für Medizinische Mikrobiologie der Ruhr-Universität, 4300 Essen, Germany.

made in BME containing 2% fetal calf serum and were inoculated intracerebrally (0.03 ml) into five animals per dilution. The  $MLD_{50}$  was calculated by the method of Reed and Muench (33).

**Buffer solutions.** Bovine albumin borate (BAB) buffer was composed of 0.12 M NaCl, 0.05 M borate, and 0.4% bovine serum albumin (Fraction V; Nutritional Biochemicals, Cleveland, Ohio), pH 9.0. Phosphate buffer was composed of 0.15 M NaCl and 0.2 M phosphate, pH 6.4. NTE buffer was composed of 0.13 M NaCl, 0.001 M ethylenediaminetetraacetate (EDTA), and 0.05 M tris(hydroxymethyl)amino-methane (Tris)-chloride, pH 7.8. Phosphate-buffered saline (PBS) was the solution described by Dulbecco and Vogt (5).

**CF test.** The microtechnique described previously (39) was used for titration of CF antigens. The reciprocal of the highest dilution of antigen showing partial reaction was taken as the titer [complement-fixing units (CFU)/0.05 ml].

**Hemagglutination.** For hemagglutination, adult goose erythrocytes (GE) were used (12). Blood was preserved in equal parts of Alsever's solution and was stored at 4 C. For each experiment, the cells were washed four times with PBS and taken up as a 0.25% suspension in phosphate buffer (pH 6.4).

The microtechnique described by Sever (37) was used. All solutions were kept in an ice-water bath. Serial twofold dilutions of the virus suspension (0.05 ml) in BAB buffer were made in plastic plates. The virus dilutions were kept at 0 to 4 C for 30 min. Equal volumes of GE suspension were then added to each well. The end point of the titration was read after incubation at 0 C for an additional 45 min. The reciprocal of the highest virus dilution showing partial HA was taken as the titer (HAU/0.05 ml). The PFU to HAU ratio was expressed as the number of PFU per milliliter over the number of HAU/0.05 ml. In this way, the value of this ratio was comparable to those given for other hemagglutinating viruses.

**Purification procedures.** Extracellular virus (ECV) grown in tissue culture was purified as described by Sokol et al. (39).

Virus grown in the brains of laboratory animals was purified as follows. Brains of infected mice or hamsters were homogenized in PBS in a Waring Blender to yield a 10% suspension. The homogenate was clarified by centrifugation for 70 min at  $45,000 \times g$ , and the pellets were resuspended in PBS (100-fold concentration). The concentrated virus was treated with equal volumes of Freon 113 (trifluorotrchloroethane; E. I du Pont de Nemours, Inc., Wilmington, Del.) under vigorous stirring at 4 C for 15 min on a magnetic stirrer. The mixture was then centrifuged at  $3,000 \times g$  for 20 min. The aqueous phase was tested for HA and infectivity.

**Virus concentration.** Two methods were used for virus concentration. (i) The virus was sedimented by centrifugation for 70 min at  $45,000 \times g$  and 4 C. The pellet was suspended in PBS by sonic treatment for 90 sec at 10 kc (Raytheon sonicator, model DF 101). Under these conditions, as determined by infectivity and hemagglutination titrations, about 90% of the virus was recovered. (ii) Dialysis of infectious

tissue culture fluid against Aquacide (carboxymethyl-cellulose; Calbiochem, Los Angeles, Calif.) for 24 hr at 4 C resulted in about a 50-fold concentration of the virus. Loss of infectivity or HA was not observed during this procedure.

**Release of intracellular virus (ICV).** Infected cells were suspended in buffer solution, frozen and thawed three times, and sonic-treated for 3 min at 10 kc. The suspension was clarified at  $7,000 \times g$  for 20 min.

**Inactivation of rabies virus by heat and ultraviolet light.** Purified and concentrated rabies virus preparations were inactivated by heating at 56 C or by ultraviolet irradiation in a petri dish with a Westinghouse lamp (giving a radiation of  $7 \times 10^3$  ergs  $cm^{-2} sec^{-1}$ ) for 10 min at a distance of 22.5 cm. The depth of the fluid was 0.3 mm. The preparation was agitated twice during the exposure time.

**Centrifugation in sucrose density gradients.** Rate zonal centrifugations in sucrose density gradients were performed as described by Sokol et al. (39).

**Treatment of virus hemagglutinin with various reagents.** One-milliliter amounts of concentrated ECV (HEP Flury strain) suspension were mixed with equal volumes of various reagents. The concentration of the compounds used and the temperature, pH, and time of treatment are given below. Virus was incubated with peroxide-free ether (J. T. Baker Chemical Co., Phillipsburg, N.J.),  $CHCl_3$  (Mallinckrodt Chemical Works, St. Louis, Mo.), or Freon 113 on a magnetic stirrer. For treatment with Tween 80 (polyoxyethylene sorbitan monooleate; Mann Research Laboratories, New York, N.Y.) and ether, the method described by Norrby (30) was used. Viral activities were determined in the aqueous phase separated by low-speed centrifugation. Sodium deoxycholate (DOC) and phospholipase C (both from Nutritional Biochemicals) were separated from the treated virus by filtration through a Sephadex G-200 column. Control samples, preserved or handled under the same conditions but without addition of reagent, were titrated in parallel with the treated samples for HA and, in most instances, also for infectivity. The action of trypsin (1:250; Difco) was stopped by addition of equal amounts of soybean trypsin inhibitor (Mann Research Laboratories).

**Treatment of GE with various reagents.** A 1-ml amount of a 10% suspension of washed GE in PBS was mixed with an equal volume of the reagent in the same buffer and was incubated at 37 C. The incubation time and the reagent concentrations are given below. The cells were then washed three times with PBS and diluted in phosphate buffer (pH 6.4) to give a 0.25% suspension. GE treated with receptor-destroying enzyme (RDE; neuraminidase of *Vibrio cholerae*; Mann Research Laboratories), which were subsequently used for agglutination by Sendai and rabies viruses, were taken up in the appropriate buffer solution to give a final concentration of 0.4%. Hemagglutination titrations were always done in parallel with treated and untreated cells.

## RESULTS

**Effects of temperature and pH and GE concentration on hemagglutination by rabies virus.** In a

comparison of the agglutination by rabies virus of GE with 1-day-old chick and guinea pig erythrocytes, the highest hemagglutination titers were obtained with GE. No difference was observed in the agglutination of red blood cells collected from various geese. The cells could be stored at 4 C for up to 4 months without loss of the ability to be agglutinated by rabies virus.

Highly purified and concentrated rabies virus (PM strain; 39) was used for determining the effect of varying pH values on agglutination of GE suspended in phosphate buffer at 4 C. The highest hemagglutination titer was obtained at pH 6.4 (Fig. 1). With the same virus preparation and a phosphate buffer with a pH of 6.4, we also tested the effect of various temperatures on the agglutination of GE. The maximal titer (128 HAU/0.05 ml) was observed at 0 and 4 C. At 10 C, the titer was lower (32 HAU/0.05 ml) and the hemagglutination pattern was not fully developed; at 22 and 37 C, hemagglutination was not observed. The effect of different erythrocyte concentrations on hemagglutination titers (with HEP strain) is shown in Fig. 2. The highest reproducible titers were obtained with a 0.25% red

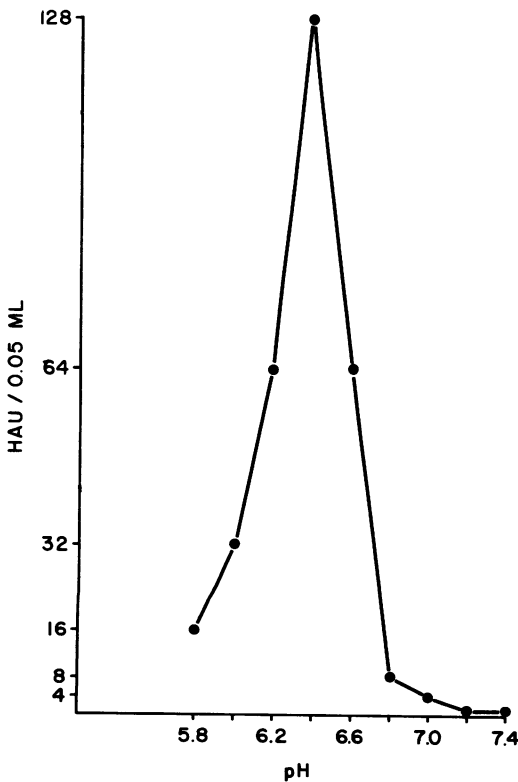


FIG. 1. Effect of pH on the agglutination of goose erythrocytes by purified rabies virus (PM strain).

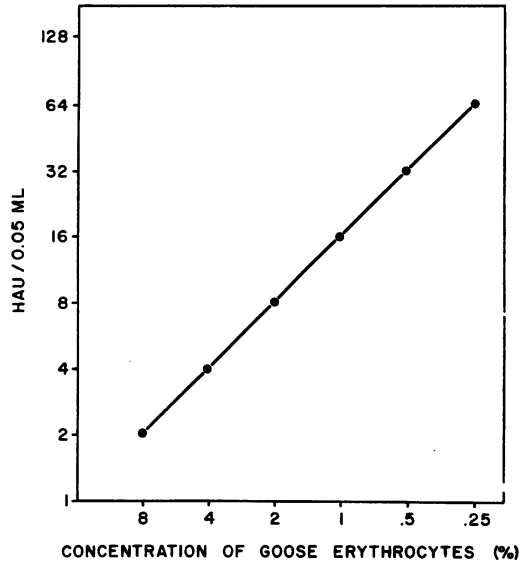


FIG. 2. Correlation between the concentration of goose erythrocytes and the hemagglutination titer of a rabies virus suspension (HEP Flury strain). The titrations were performed at 4 C and pH 6.4.

cell suspension. Therefore, the following experiments were performed at pH 6.4 and 4 C, with 0.25% GE suspension.

*Hemagglutination by different rabies virus strains and the lack of effect of the host cell species on the HA of the virus.* Since Halonen et al. (12) used only one strain of virus in their study of rabies HA, it was of interest to determine whether other rabies virus strains possess the same HA. Five strains (Table 1) grown in BHK-21 cells and in brain tissue were concentrated 10 to 30 times by the first method described in Materials and Methods and were checked for their ability to agglutinate GE.

CVS, PM, HEP, Pasteur, and ERA strains grown in tissue culture agglutinated GE, giving similar PFU to HAU ratios, but the PM strain grown in mouse and hamster brain did not agglutinate GE (Table 1).

Because the HEP Flury strain consistently yielded higher infectivity and hemagglutination titers in BHK-21 cells than in the other cell strains listed in Table 1, all further experiments were done with this virus strain propagated in BHK-21 cells.

*Reagglutination of GE pretreated with rabies virus.* The following experiment was performed to determine whether GE from which the rabies virus was eluted can be reagglutinated by exposure to homologous and heterologous virus. Rabies virus suspensions (concentrated by the first method), containing 128 HAU/0.05 ml, were

adsorbed at 4 C for 1 hr with equal volumes of 4% GE suspensions in phosphate buffer (pH 6.4). One-third of the virus did not attach to the erythrocytes. The agglutinated GE were then collected by low-speed centrifugation, and the adsorbed virus was eluted into the phosphate buffer at 37 C for 30 min. As will be shown later, under these conditions, almost all of the virus was released from the erythrocytes. After being washed three times in PBS, GE were suspended in the phosphate buffer to a final concentration of 0.25%. Treated and untreated GE were then exposed to rabies or Sendai virus, respectively. Pretreatment with rabies virus did not affect the ability of GE to be reagglutinated by rabies or Sendai viruses (Table 2).

*Lack of effect of RDE on the agglutination of GE by rabies virus.* One-milliliter amounts of an 8% suspension of GE in PBS were mixed with equal volumes of RDE of increasing concentrations. The mixtures were incubated with occasional shaking for 2.5 hr at 37 C. The GE were then washed three times with PBS, divided into two equal parts, and suspended either in PBS or in phosphate buffer (pH 6.4) for hemagglutination

TABLE 1. Agglutination of goose erythrocytes by five different rabies virus strains grown in BHK-21 cells and by the PM strain grown in five different host cells

Virus strain	HAU per 0.05 ml <sup>a</sup>	PFU per ml
CVS grown in BHK-21 cells <sup>b</sup>	32	$2 \times 10^7$
PM grown in BHK-21 cells <sup>b</sup>	128	$10^8$
HEP grown in BHK-21 cells <sup>b</sup>	256	$7 \times 10^8$
Pasteur grown in BHK-21 cells <sup>b</sup>	32	$4 \times 10^7$
ERA grown in BHK-21 cells <sup>b</sup>	8	$8 \times 10^6$
PM grown in BHK-21 cells <sup>b</sup>	32	$5 \times 10^7$
WI-38 cells <sup>b</sup>	32	$6 \times 10^7$
Nil-2 cells <sup>b</sup>	64	$9 \times 10^7$
Mouse brain <sup>c</sup>	<1	$4 \times 10^7$
Hamster brain <sup>c</sup>	<1	$2 \times 10^7$

<sup>a</sup> Hemagglutination was performed at pH 6.4 and 0 C, with either concentrated infectious tissue culture fluid free from serum or partially purified and concentrated brain-grown virus.

<sup>b</sup> Infectious tissue culture fluid was harvested between 72 and 84 hr after infection. The input multiplicity of infection was between 10 and 30 PFU per cell.

<sup>c</sup> Virus-containing mouse and hamster brains were harvested 96 to 120 hr after infection.

by Sendai and rabies viruses (concentrated by the first method), respectively. Hemagglutination titers of Sendai virus were read after incubation for 1 hr at 20 C; hemagglutination titers of rabies virus were read after 1 hr at 0 C (see Fig. 3). RDE, at a concentration of 12.5 units/ml or lower, did not affect the agglutination of the GE by either virus. At higher concentrations of RDE, a marked decrease in the agglutination of GE by Sendai virus was observed. On the other hand, the agglutination of GE by rabies virus was not affected,

TABLE 2. Effect of pretreatment with rabies virus on agglutination of goose erythrocytes

Virus	Hemagglutination titer (HAU per 0.05 ml)	
	Untreated goose erythrocytes	Erythrocytes pretreated with rabies virus
Rabies (HEP Flury strain) <sup>a</sup> . . . . .	128	128
Sendai <sup>b</sup> . . . . .	2,048	2,048

<sup>a</sup> Hemagglutination reaction performed at 4 C.

<sup>b</sup> Hemagglutination reaction carried out at 22 C.

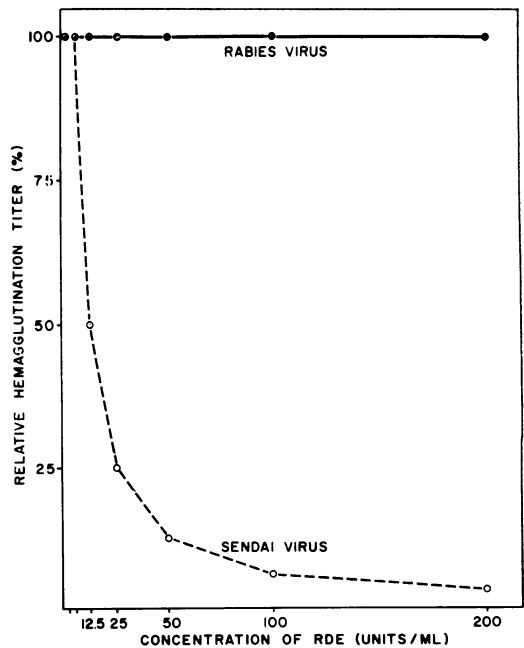


FIG. 3. Effect of receptor-destroying enzyme (RDE) on the agglutination of goose erythrocytes by Sendai and rabies (HEP Flury strain) viruses. The titers of the original suspensions were 1,024 and 128 HAU per 0.05 ml for Sendai and rabies viruses, respectively.

even at the highest concentration of RDE. The efficiency of the RDE treatment was determined by assay for the presence of free neuraminic acid in the supernatant fluids after removal of the GE, according to the method of Warren (40). We found that free neuraminic acid increased in parallel with the amount of RDE used.

*Attempts to characterize the receptors for rabies virus by treatment of GE with various compounds.* Experiments aimed at the characterization of the nature of the receptors for rabies virus were not conclusive. None of the substances listed in Table 3 had an effect on the agglutination of GE by rabies virus; therefore, the nature of the receptor(s) for rabies virus present in GE remains uncertain.

*Relationship between infectivity and HA of rabies virus.* To investigate whether a linear relationship exists over a wide concentration range between the infectivity and the HA of the virus, crude (concentrated by both methods) as well as highly purified virus was titrated by plaque assay and was assayed for HA. The infectivity was proportional to HA, provided that the infectivity of the virus sample was higher than or equal to  $10^6$  PFU/ml (Fig. 4).

*Failure to demonstrate "soluble" hemagglutinin in infectious tissue culture fluid.* The following experiment was carried out to elucidate whether HA is associated exclusively with the virions or whether the infected cells produce "soluble" hemagglutinins which sediment more slowly than the virions.

Clarified infectious tissue culture fluid was concentrated by the second method without prior centrifugation. A sample of the same virus material before dialysis was centrifuged at  $45,000 \times g$  for 70

TABLE 3. Compounds not affecting the agglutination of goose erythrocytes by rabies virus<sup>a</sup>

Compound	Concn
EDTA	$5 \times 10^{-2}$ M
Trypsin	0/2%
Pronase	0/5%
KIO <sub>4</sub>	$5 \times 10^{-3}$ M
2-Mercaptoethanol	$5 \times 10^{-3}$ M
p-Chloromercuribenzoate	$5 \times 10^{-3}$ M
HgCl <sub>2</sub>	$5 \times 10^{-4}$ M
Iodoacetic acid	$5 \times 10^{-3}$ M
RDE	100 units/ml
Phenylmethyl-sulfonylfluoride	$10^{-3}$ M

<sup>a</sup> Cells were treated for 60 min at pH 7.3 and 37 C. Treatment with trypsin was performed for 5 hr; treatment with p-chloromercuribenzoate was at pH 8.5.

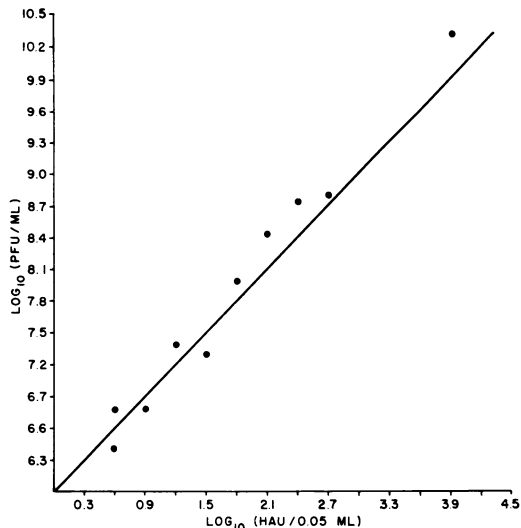


FIG. 4. Relationship between infectivity and hemagglutinating activity of extracellular rabies virus (HEP Flury strain). Symbols: ●, mean values of 10 determinations; standard deviation,  $\pm 0.3$  to  $\pm 0.5$  log<sub>10</sub> units, solid line, ratio of PFU to HAU =  $10^6$ .

min to sediment the bulk of the virus, and the supernatant fluid was concentrated 50-fold in the same way. The centrifuged sample contained about seven times less infectious virus than the sample concentrated directly by dialysis. Both samples were fractionated by rate zonal centrifugation in a 10 to 50% (w/w) sucrose gradient, and the collected fractions were assayed for infectivity and HA. Only one peak of HA, coinciding with the peak of infectivity, was found in both gradients (Fig. 5). Of interest is the observation that the PFU to HAU ratios of fractions 5 to 7 were about  $10^6$ , whereas the same ratios of fractions 8 to 10 approached  $10^5$ . Some lighter hemagglutinating material, which differs only slightly from infectious virus in sedimentation rate, may be present in both virus samples (39).

*Release of infectious and hemagglutinating virus from infected cells.* The experiments described thus far were carried out with virus harvested at 72 to 96 hr after infection. The dynamics of the release of infectious virus and viral hemagglutinin into the tissue culture fluid was investigated to determine whether a "soluble" hemagglutinin is released into the fluid soon after infection and then is destroyed during prolonged incubation. Monolayer cultures of BHK-21 cells in milk dilution bottles ( $10^7$  cells/bottle) were infected at an input multiplicity of 30 PFU/cell. Samples for assay of infectivity and assay of HA were collected at intervals after infection. The amount of virus released into the medium increased logarithmic-

ally from 12 to 48 hr, reached a peak at 72 hr after infection, and thereafter remained constant (Fig. 6). After its first appearance at 24 hr post-infection, HA increased in parallel with infectivity up to 96 hr. During the next 4 days, release of infectious virus decreased gradually, whereas the level of HA remained almost constant. The results of this experiment indicated that rabies virus, inactivated at 37 C, is still capable of agglutinating GE. Similarly, the storage of rabies-infected tissue culture fluid at 4 C for 8 weeks

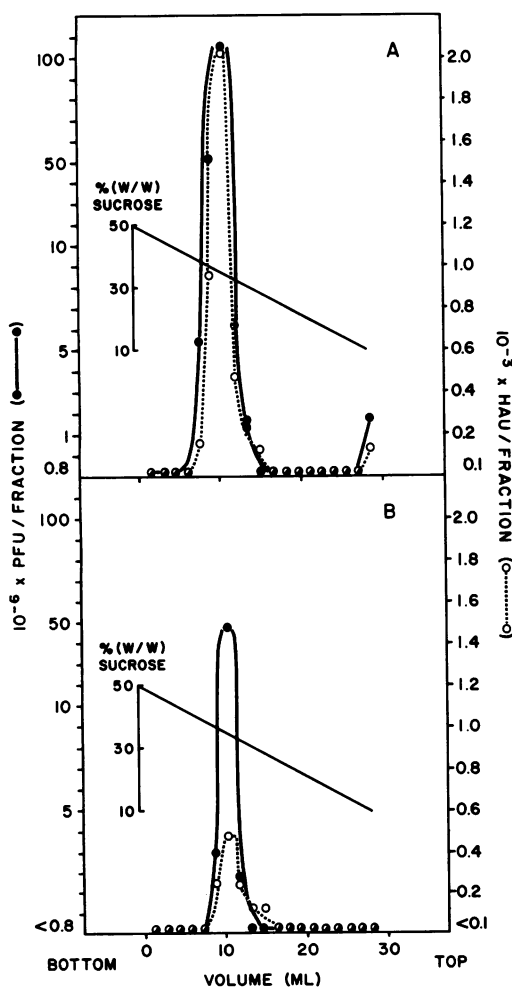


FIG. 5. Rate zonal centrifugation of extracellular rabies virus preparation (HEP Flury strain). Both samples of infectious tissue culture fluid were concentrated 50-fold by dialysis against Aquacide before sucrose density gradient centrifugation. (A) Virions not removed. (B) Before concentration, the bulk of the virions were removed by high-speed centrifugation. The gradients were centrifuged in an SW 25.1 rotor of a Spinco centrifuge for 90 min at  $61,000 \times g$ .

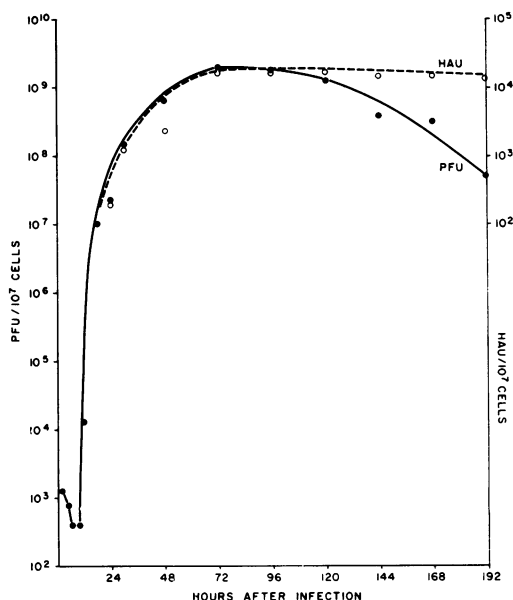


FIG. 6. Release of rabies virus (HEP Flury strain) from BHK-21 cells. The experiment was carried out under conditions of one-step growth. After virus adsorption, the cultures were treated with anti-rabies serum. The serum was subsequently removed by repeated washing with PBS.

did not affect its HA, whereas infectivity decreased 25 times during this time.

*Comparison of hemagglutinating activity of ECV and ICV.* When the amount of ICV was determined at different intervals after infection by infectivity titration and HA tests in extracts from  $10^7$  cells, hemagglutinating activity was not detectable in spite of a high concentration of infectious virus ( $10^8$  PFU/ml, 72 hr after infection). To determine whether ICV exhibits any HA, extracts from larger amounts of infected cells were assayed for content of infectious and hemagglutinating virus. ECV from the same cultures was tested in parallel. These experiments (Table 4) showed that the ICV preparation contained little HA, although the concentration of infectious virus in the ICV preparation was higher than in the ECV preparation.

Experiments were performed to determine whether the difference in HA between ECV and ICV is due to the presence of hemagglutination inhibitors in noninfected or rabies virus-infected cells. First, the influence of the binding of  $Ca^{++}$  by EDTA on the HA of ECV and ICV was tested.  $Ca^{++}$  was reported to be essential for the interaction of influenza virus with certain inhibitors (23). Second, ICV was treated with Freon 113 to determine whether this treatment would "un-

mask" the HA of ICV by removal of potential inhibitors, as is found with other hemagglutinating viruses (9, 35). Third, extracts from  $8 \times 10^8$  non-infected and rabies virus-infected BHK-21 cells (harvested 72 hr after infection) were prepared in the same manner as the ICV suspensions and were checked for inhibitory activity on hemagglutination by ECV.

The binding of  $\text{Ca}^{++}$  by EDTA neither promoted nor decreased hemagglutination by rabies virus (Table 5; also see Table 6). Treatment with Freon 113 decreased infectivity of the virus 10-fold, but did not affect HA. Extracts from noninfected or infected BHK-21 cells had no influence on the HA of ECV.

*Effect of various agents on the HA and infectivity of rabies virus.* To elucidate the chemical

TABLE 4. *Infectivity and hemagglutinating activity of extracellular and intracellular rabies viruses (HEP Flury strain)*

Prepn	Expt 1 <sup>a</sup>			Expt 2 <sup>b</sup>		
	PFU per ml	HAU per 0.05 ml	PFU per HAU	PFU per ml	HAU per 0.05 ml	PFU per ml
ICV.....	$10^{8.5}$	$10^{0.6}$	$10^{7.9}$	$10^{7.8}$	$10^{0.3}$	$10^{7.5}$
ECV.....	$10^{7.5}$	$10^{1.2}$	$10^{6.3}$	$10^{8.0}$	$10^{2.7}$	$10^{6.3}$

<sup>a</sup> ICV:  $6 \times 10^8$  cells were extracted with 1 ml of 0.5 EDTA-Tris, pH 7.8. ECV: nonconcentrated infective tissue culture fluid; the culture was harvested 72 hr after infection; input multiplicity of infection, 30 PFU per cell.

<sup>b</sup> ICV:  $1.8 \times 10^9$  cells were extracted with 3 ml of 0.5 EDTA-Tris, pH 7.8. ECV: the virus was concentrated 30-fold by high-speed centrifugation of the infectious tissue culture fluid; the culture was harvested 84 hr after infection; input multiplicity of infection, 20 PFU per cell.

nature of rabies virus hemagglutinin, we investigated the effect of various agents on the HA of rabies virus concentrated by the first method (Table 6). As mentioned before, EDTA had neither an enhancing nor an inhibitory effect on the HA or infectivity of the virus. Proteolytic enzymes, such as trypsin and Pronase, completely destroyed HA and infectivity.  $\text{HgCl}_2$  (Fisher Scientific Co., Fairlawn, N. J.) and *p*-chloromercuribenzoate (pCMB; Calbiochem), compounds blocking the sulfhydryl groups, also destroyed HA and infectivity. Iodoacetic acid (Calbiochem), however, destroyed the infectivity but not the HA of the virus. All lipid solvents and emulsifiers tested (ether, chloroform, Freon 113, phospholipase C, Tween 80, DOC), as well as  $\beta$ -propiolactone (BPL), inactivated both HA and infectivity.  $\text{KIO}_4$  (J. T. Baker Chemical Co.), 2-mercaptoethanol (Calbiochem), and dithioreitol (Calbiochem) did not affect the HA, but the first compound suppressed the infectivity of the virus. Irradiation by ultraviolet light selectively destroyed the infectivity without affecting hemagglutination, whereas heating at 56 C destroyed both HA and infectivity.

*Rabies virus adsorption onto and elution from GE.* When GE agglutinated at 4 C were transferred to 20 C, the hemagglutination pattern disappeared within 10 to 15 min, suggesting that at higher temperatures the virus is eluted from the red blood cells. The following experiments were performed to check this possibility.

Preparations of rabies virions concentrated by the first method were diluted with equal volumes of BAB buffer at 4 C. The diluted virus suspensions were then mixed with equal volumes of 10% GE suspension in phosphate buffer (pH 6.4). The mixtures were kept at 4 C for 1 hr and centrifuged at low speed; the infectivity, CF titers, and HA of

TABLE 5. *Lack of effect of EDTA, fluorocarbon, and extracts from normal and rabies virus-infected BHK 21 cells on agglutination of goose erythrocytes by intracellular and extracellular rabies viruses (HEP Flury strain)<sup>a</sup>*

Virus activity	ICV <sup>b</sup>			ECV <sup>c</sup>		
	Untreated	Diluted 1:2 with 1.0 M EDTA	Treated with Freon 113	Diluted 1:2 with PBS	Diluted 1:2 with extract from non-infected cells	Diluted 1:2 with extract from rabies virus-infected cells
PFU per ml.....	$10^{8.5}$	$10^{8.3}$	$10^{7.6}$	$10^{7.7}$	$10^{7.6}$	$10^{8.1}$
HAU per 0.05 ml.....	$10^{0.6}$	$10^{0.3}$	$10^{0.3}$	$10^{1.5}$	$10^{1.5}$	$10^{1.5}$
PFU per HAU.....	$10^{7.9}$	$10^{8.0}$	$10^{7.3}$	$10^{6.2}$	$10^{6.1}$	$10^{6.6}$

<sup>a</sup> The virus was treated with EDTA or with cellular extracts at 20 C for 30 min. Treatment with Freon 113 was performed at 4 C for 15 min on a magnetic stirrer.

<sup>b</sup> See Table 2, experiment 1.

<sup>c</sup> Harvested 72 hr after infection and concentrated 30-fold by high-speed centrifugation.

the supernatant fluids were determined. The virus was then eluted in two cycles from the erythrocytes into phosphate buffer (*pH* 6.4) at 37 C for 30 min. About 90% of virus was adsorbed to the erythrocytes and about 60% was recovered in the eluates (Table 7). Adsorption or elution differences between purified and crude virus preparations were not observed.

To determine whether the virus can be quantitatively eluted at temperatures lower than 37 C, samples of a virus suspension were adsorbed with different amounts of erythrocytes. The conditions of adsorption were similar to those described in the previous experiment. Without separating the agglutinated erythrocytes, the virus was then

eluted at 4, 22, and 37 C. The GE were removed by low-speed centrifugation, and the HA in the supernatant fluids was determined. A parallel control experiment was done to determine the proportion of the virus adsorbed to the erythrocytes (Fig. 7). The virus was quantitatively eluted during prolonged incubation both at 4 and 22 C. The results also indicated that a relatively large number of erythrocytes are required for adsorption of all virus particles. The relationship between the erythrocyte concentration and the efficiency of adsorption of the virus and its elution is shown in Fig. 8. In this experiment, the agglutinated erythrocytes were separated by low-speed centrifugation before elution of the virus.

TABLE 6. *Effect of various agents on the hemagglutinating activity and infectivity of rabies virus (HEP Flury strain)*

Agent and conditions of treatment	Final concn of the compound	HAU per 0.05 ml	PFU per ml
EDTA, 60 min, <i>pH</i> 7.3, 37 C	$5 \times 10^{-2}$ M	64	$6 \times 10^7$
Control		64	$6 \times 10^7$
Trypsin, 60 min, <i>pH</i> 7.3, 37 C	0.2%	<1	<10 <sup>3</sup>
Control		32	$6 \times 10^7$
Pronase, 60 min, <i>pH</i> 7.3, 37 C	0.5%	<1	<10 <sup>3</sup>
Control		128	$9 \times 10^7$
KI <sub>0</sub> , 60 min, <i>pH</i> 7.3, 37 C	$5 \times 10^{-3}$ M	32	<10 <sup>3</sup>
Control		32	$4 \times 10^7$
2-Mercaptoethanol, 60 min, <i>pH</i> 7.3, 37 C	$5 \times 10^{-3}$ M	64	$2 \times 10^8$
Control		64	$9 \times 10^7$
Dithiothreitol, 60 min, <i>pH</i> 7.3, 37 C	$5 \times 10^{-3}$ M	32	Not done
Control		32	Not done
HgCl <sub>2</sub> , 60 min, <i>pH</i> 7.3, 22 C	$5 \times 10^{-4}$ M	<1	<10 <sup>3</sup>
Control		64	$9 \times 10^7$
pCMB, 60 min, <i>pH</i> 8.5, 37 C	$5 \times 10^{-3}$ M	<1	<10 <sup>3</sup>
Control		32	$4 \times 10^7$
Iodoacetic acid, 60 min, <i>pH</i> 7.3, 37 C	$5 \times 10^{-3}$ M	128	<10 <sup>3</sup>
Control		64	$2.2 \times 10^7$
Ether, 30 min, <i>pH</i> 7.3, 4 C	50%	<1	<10
Control		64	$5 \times 10^7$
Tween 80, 30 min, <i>pH</i> 7.3, 4 C; then ether, 15 min, <i>pH</i> 7.3, 4 C	0.125%	2	<10
Control	33%	64	$5 \times 10^7$
Freon 113, 30 min, <i>pH</i> 7.3, 4 C	50%	16	$7 \times 10^8$
Control		64	$5 \times 10^7$
CHCl <sub>3</sub> , 30 min, <i>pH</i> 7.3, 4 C	50%	<1	<10
Control		64	$5 \times 10^7$
Phospholipase C, 20 min, <i>pH</i> 7.3, 4 C	0.5%	<1	<10
Control		128	$4 \times 10^7$
DOC, 10 min, <i>pH</i> 7.3, 4 C	0.5%	<1	<10
Control		128	$4 \times 10^7$
BPL, 20 hr, <i>pH</i> 7.6, 4 C	0.025%	<1	<10
Control		128	$7 \times 10^7$
Heating, 30 min, <i>pH</i> 7.3, 56 C		<1	<10 <sup>3</sup>
Control		64	$5 \times 10^7$
Ultraviolet light, 10 min, <i>pH</i> 7.3, 22 C		128	<10
Control		128	$7 \times 10^7$



TABLE 7. Adsorption onto and elution from goose erythrocytes of rabies virus (HEP Flury strain)<sup>a</sup>

Expt. no.	Activity	Original virus suspension	Supernatant fluid after adsorption	First elution	Second elution
1	PFU per ml	10 <sup>7.46</sup> (100%)	10 <sup>6.62</sup> (15%)	10 <sup>7.21</sup> (57%)	10 <sup>3.85</sup> (0.03%)
	HAU per ml	10 <sup>3.26</sup> (100%)	10 <sup>1.90</sup> (4.4%)	10 <sup>3.11</sup> (70%)	<10 <sup>1.30</sup> (<1%)
	CFU per ml	10 <sup>3.11</sup> (100%)	10 <sup>1.90</sup> (6.3%)	10 <sup>2.81</sup> (50%)	<10 <sup>1.30</sup> (<1%)
2	PFU per ml	10 <sup>7.12</sup> (100%)	10 <sup>6.04</sup> (8.5%)	10 <sup>6.9</sup> (61%)	10 <sup>3.60</sup> (0.03%)
	HAU per ml	10 <sup>2.98</sup> (100%)	10 <sup>1.90</sup> (8.3%)	10 <sup>2.68</sup> (50%)	<10 <sup>1.30</sup> (<1%)
	CFU per ml	10 <sup>3.41</sup> (100%)	10 <sup>1.90</sup> (3.1%)	10 <sup>3.26</sup> (71%)	10 <sup>1.60</sup> (0.15%)

<sup>a</sup> Experiment 1: concentrated crude virus preparation was used. Experiment 2: purified virus suspension was used. The recovery rates (%) at each stage of the experiment are given in parentheses.

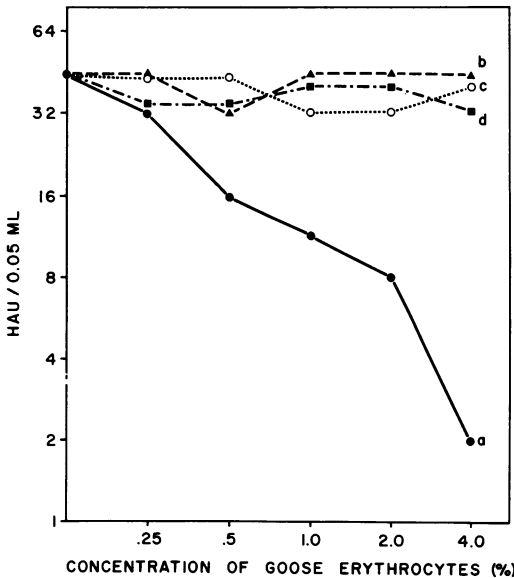


FIG. 7. Effect of erythrocyte concentration used for adsorption and temperature of elution on the recovery of rabies virus in the eluate. Nonattached rabies virus (a) after adsorption at 4 C for 60 min; (b) eluted at 4 C for 180 min; (c) eluted at 22 C for 45 min; (d) eluted at 37 C for 45 min.

DISCUSSION

Rabies viruses agglutinate GE at 4 C and at a pH lower than 6.8. In this respect, they resemble arboviruses. All rabies virus strains tested agglutinated GE and had the same PFU to HAU ratio. To obtain positive hemagglutination, the level of virus must be sufficiently high (10<sup>6</sup> PFU/ml or higher) and the virus must be grown in tissue culture, rather than in brain tissue, in the absence of hemagglutination inhibitors.

All rabies virus strains tested exhibited HA, which is in contrast with poxviruses (6), echoviruses (7, 25), type B Coxsackie viruses (34), and adenoviruses (2), in which erythrocytes are agglutinated only by some strains within a serotype.

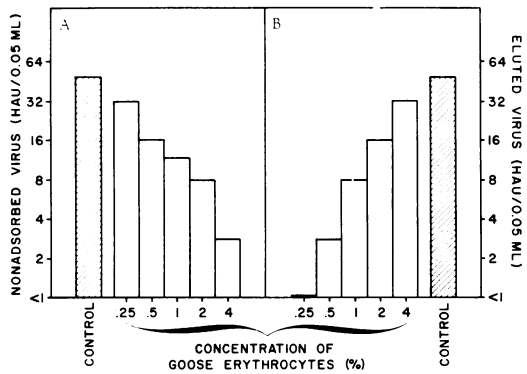


FIG. 8. Effect of concentration of red blood cells on the adsorption of rabies virus (HEP Flury strain) onto goose erythrocytes (GE) and on its subsequent elution. (A) The virus was adsorbed on GE at 4 C for 60 min in phosphate buffer, pH 6.4. The erythrocytes were then collected by low-speed centrifugation, and the hemagglutinating activity of the supernatant fluid was determined. Control: no erythrocytes added. (B) The virus was eluted at 37 C for 40 min into the original volume of phosphate buffer, pH 6.4. Control: original virus suspension heated at 37 C for 40 min.

The hemagglutinin is evidently coded by the viral genome rather than by the cellular genome, because the HA of the rabies virus is independent of the cell type in which the virus is propagated; thus, the cellular genome does not participate in the synthesis of rabies virus hemagglutinin, as is the case with vaccinia virus hemagglutinin (16).

The HA of rabies virus is associated with the virion, and "soluble" hemagglutinin was not detected either in extracts of infected cells (39) or in infectious tissue culture fluid. In this respect, rabies virus seems to differ from poxviruses (28), measles virus (31), myxoviruses (11), and reovirus (43). The replication of these viruses is accompanied by the appearance of noninfectious viral hemagglutinin which sediments at a lower rate than the virion. "Soluble" hemagglutinin, which has been successfully released from myxoviruses

(17, 20, 38), measles virus (30, 41), and rubella virus (8) by treatment with lipid solvents, cannot be liberated from rabies virus by this method.

When compared, infectivity and HA of rabies virus preparations exhibited a linear relationship; thus, under certain circumstances, the HA test can replace infectivity titration. With rabies virus, myxoviruses (16), and echoviruses (3, 25), hemagglutination becomes positive when the level of infectious virus exceeds  $10^6$  PFU per ml. In contrast, with reovirus and measles virus, hemagglutination becomes positive when the level of infectious virus approaches  $10^5$  to  $10^6$  (14, 26, 31). It should be noted that the experimental conditions used for both HA and infectivity tests strongly influenced the infectivity to HA ratio.

Halonen et al. (12) found that the release of rabies virus hemagglutinin from infected cells occurred later than the release of infectious virus and that the peaks were reached at different times after infection. This finding was not confirmed because, under the experimental conditions used in this study, the hemagglutinin was released in parallel with the infectious virus.

ICV released from infected cells by freezing and thawing, and then by sonic treatment, showed much lower HA (PFU to HA ratio from  $10^{7.3}$  to  $10^{8.0}$ ) than ECV; however, no HA inhibitor similar to the one described by Schmidt et al. (35) for enteroviruses was detected. It is possible, therefore, that ECV acquired HA during budding from transformed cell membranes (18, 19). It cannot be determined from available data whether ICV represents an infectious "precursor" of the mature, hemagglutinating virion. We reported previously (39) that some of the partially purified preparations of ICV (HEP Flury strain) had a PFU to HAU ratio comparable to that of ECV. Infected cells may contain variable amounts of virus particles which exhibit HA after they bud from the cell surface and are released into vacuoles (19). Consequently, the PFU to HAU ratio of ICV preparations would be subject to variation. A comparative study of ICV and ECV was carried out with only one virus strain (HEP Flury). It may be that these differences are less pronounced with other virus strains.

The virus which adsorbed to GE at 0 C and at pH 6.4 could be eluted from the agglutinated erythrocytes over a wide temperature range, providing that sufficient time was allowed for elution. Because RDE had no effect on the receptors for rabies HA, it is possible that an enzyme other than neuraminidase may be instrumental in causing elution of the virus at low temperatures. Adsorption onto and elution from GE can thus be used for concentration and partial purification of ECV.

Rabies virus has tentatively been classified (27) as a myxovirus (subgroup II). The properties of its inner component (ribonucleoprotein) are similar to the properties of the inner component of this myxovirus subgroup (19; F. Sokol, *unpublished data*), but its capsid, in contrast to the capsid of myxoviruses, does not contain neuraminidase (10) and has no affinity for mucoproteins.

The hemagglutinating component of the virion is sensitive to heating at 56 C and to treatment with proteolytic enzymes, lipid solvents, BPL, and compounds blocking sulfhydryl groups, such as pCMB and  $HgCl_2$ . Results obtained after treatment with  $KIO_4$  (Table 6) may be explained by the fact that  $KIO_4$  reacts primarily with polysaccharides by oxidation of glycol groups, which may lead to destruction of infectivity without affecting HA. Compounds capable of cleaving the —S—S— bonds, such as 2-mercaptoethanol and dithiothreitol, had no effect on HA and infectivity. These results indicate that, since the rabies hemagglutinin is probably a protein or lipoprotein, the SH-groups may play an important role in the attachment of rabies virus to the erythrocytes. The receptor of the rabies virus hemagglutinin does not seem to be either a protein or a polysaccharide. It may be a lipid or lipoprotein, as receptors of hemagglutinating enteroviruses were shown to be by Philipson and Bengtsson (32). More detailed experiments are necessary, however, to elucidate the chemical nature of rabies virus receptors.

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