weeks after intracardial injection of *M. tuberculosis* strain $H_{37}R_v$ was used to determine the immunoglobulin class of antibody responsible for the HA and HL reaction. Only 19S immunoglobulin could be associated with this antibody activity.

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Studies on Hemagglutination by Rubella Virus.* (32560)

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The discovery by Stewart *et al*(1) of an hemagglutinin which appeared in BHK21 cultures after infection began a new period in the immunological study of rubella: This discovery was based on the removal of an hemagglutinin-inhibitor both from the fetal calf serum used to grow the virus and from the sera to be tested for specific hemagglutinin-inhibiting antibody.

Recently we reported the preparation of hemagglutinin (HA) from infected BHK21 culture fluids(2) by treatment of tissue culture fluids with sodium ethylenediaminetetraacetate (EDTA), which separates HA from inhibitor. We presented evidence for the hypothesis that the attachment between HA and inhibitor depended on the presence of divalent cations and that EDTA released inhibitor by its binding action on cations.

In this paper, we report a more detailed study of rubella virus (RV) HA prepared by this method.

Materials and methods. Virus: The RA 27/3 strain(3) in the 18th to 24th passage

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in BHK 21/13S cells(4) was used throughout these experiments.

Cell culture: BHK21/13S cell culture, grown in monolayer in Stoker and McPherson's ETC medium + 10% fetal calf serum (5), was used. After inoculation with virus, the cells were incubated at 35°C either in monolayer culture with Eagle's Basal Medium (BME) and 2% fetal calf serum, or in suspension culture (10^6 cells/ml) with ETC and 5% fetal calf serum.

HA production: The cells were inoculated in monolayer, or in suspension in a flask stirred by a magnet, at a multiplicity of 1 plaque-forming unit (PFU) per cell. Tissue culture fluids were harvested at 3, 4, or 5 days, depending on the results obtained by testing samples. In general, suspension cultures yielded higher titers of hemagglutinin. One-tenth volume of a 0.1 M disodium ethylenediaminetetraacetate (EDTA) solution was added to tissue culture fluids, making a .01 M solution. After 30 minutes at 4°C, the fluid was centrifuged for 2½ hours at 32,000 *g* in a Servall centrifuge. The pellet was resuspended with phosphate-buffered saline (PBS) containing .001 M EDTA, in one-fifth or one-tenth the original volume.

Red blood cells: Newly hatched chickens were bled, using sterile Alsever's solution as anticoagulant, and the blood was stored at 4°C. Before use, the red blood cells were washed 3 times with PBS (pH 7.3) and suspended in the same solution at a concentration of 0.25% (V/V).

Hemagglutination test: 2-fold serial dilutions of HA were made in PBS, pH 7.3, in volumes of 0.5 ml. Red blood cells (0.5 ml of 0.25% suspension) were then added to each HA dilution and, after thorough mixing, the tubes were incubated at 4°C for 2 hours. The titers were expressed as the highest initial dilution showing complete agglutination.

Hemagglutination-inhibition test: Serial 2-fold dilutions of serum specimens were prepared in 0.25 ml volumes of PBS, to which 4 units of HA in 0.5 ml PBS were added. The sera had previously been tested with kaolin and 50% red cells to remove inhibitor (1). After incubation of this mixture for 2 hours at 4°C, 0.25 ml of 0.5% red blood cells was added and the entire tube reincubated at 4°C for 2 hours.

Results. Preparation of HA: 2 simple methods were developed for the preparation of HA. In the first, tissue culture fluids (TCF) were treated with EDTA (see *Methods*) and, in the second, with Tween-ether according to the procedure of Norrby (6). With Tween-ether treatment, maximum titers of 64 HA units/ml were obtained. Because the EDTA method involved a concentration step, it was not difficult to obtain titers in the range of 640 HA units/ml. The specificity of the two HA antigens was confirmed by the demonstration that sera from convalescent rubella cases and rubella syndrome infants inhibited their agglutination of red cells, while acute sera and sera from normal infants did not.

The concentration of EDTA added to TCF was varied over a wide range, and the TCF was held before centrifugation either for 2 hours or overnight. As shown in Table I, increasing the concentration of EDTA over 0.01 M or keeping the fluids overnight did not improve the yield of HA.

TABLE I. Effect of EDTA Concentration and Treatment Time on Release of Hemagglutinin.

EDTA concentration	Treatment time 2 hr	18 hr
1/10 saturation	1:256*	1:128
.1 M	1:256	1:128
.01 M	1:256	1:128
.001 M	<1:4	1:8
.0001 M	<1:4	<1:4
Control	<1:4	<1:4

* HA/ml obtained by 5-fold concentration of tissue culture fluid by centrifugation at 32,000 *g* for 2 hr, followed by re-suspension in one-fifth the original volume.

Relation of RV hemagglutinin to viral infectivity. In Table II are given the results of an experiment in which TCF was harvested daily, titrated for infectivity, and treated with either EDTA or Tween-ether to prepare HA. Although the HA titers did not reach a peak until 5 days, infectivity was highest at 3 days. Thus, viral and HA titers were not parallel. Nevertheless, when an HA-positive harvest prepared by EDTA treatment was placed on a sucrose density gradient (Fig. 1), the HA was found only in the density frac-

TABLE II. Production of Rubella Virus Hemagglutinin in BHK21 Culture.

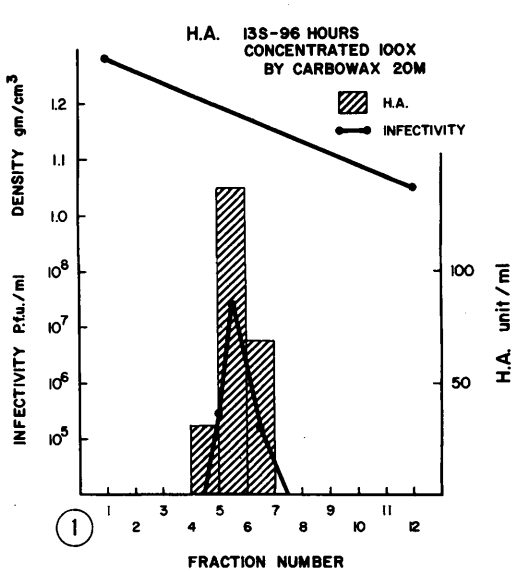
Days after infection	Exp. 1 Tween-ether*	Exp. 1 EDTA† (conc. 5×)	Exp. 1 Infectivity (PFU/ml)	Exp. 2 Tween-ether	Exp. 2 EDTA (conc. 10×)
1	<1:2‡	<1:2	$10^{5.8}$	<1:2	N.T.
2	1:2	<1:2	$10^{6.5}$	1:2	N.T.
3	1:8	1:16	$10^{7.3}$	1:4	N.T.
4	1:32	1:128	$10^{6.9}$	1:64	1:320
5	1:64	1:128	$10^{6.4}$	1:64	1:640

* Direct treatment of tissue culture fluid with Tween-ether.
† Addition of EDTA to tissue culture fluid followed by centrifugation (see *Methods*).
‡ Titer of HA.

tions which contained infectious virus, *i.e.*, about 1.19 g/cm³.

Characteristics of HA. The general properties of HA prepared by the EDTA or Tween-ether methods are summarized in Table III. In both types of HA preparations, agglutination occurred with 1-day-old chicken cells, adult chicken cells or sheep cells, but not with human or guinea-pig cells. Cells from 1-day-old chicks were the most sensitive. Titers were highest when the agglutination was performed at 4°C, but pH did not seem to be critical.

EDTA-prepared HA was sedimented by centrifugation for 2½ hours at 32,000 *g* and was not filtrable through a millipore filter of 100 mμ pore size. The HA remained stable for at least one month at −20°C and for one week at 4°C, but was destroyed by treatment for 1 hour with ether. 90 percent of the HA titer was lost after heating EDTA antigen at 56°C for 15 minutes, but at 37°C no substantial loss had occurred after 5 hours. One-day old chick cells, tested previously with receptor-destroying enzyme (RDE) of V. cholera which rendered them inagglutinable by



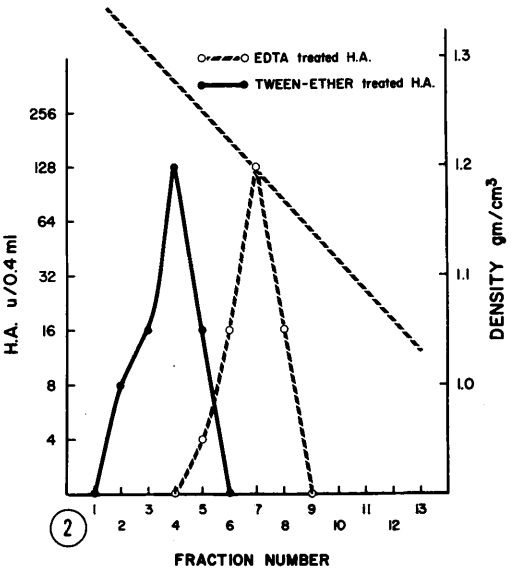


FIG. 1. Distribution of hemagglutinin and infectivity on a preformed 5 to 60% sucrose density gradient centrifuged at 35,000 rpm for 9 hr in the SW 39 rotor.

FIG. 2. Sucrose density gradient centrifugations of EDTA-released rubella HA and EDTA-released Tween-ether-treated rubella HA shown on same graph. Gradients consisted of 5-60% sucrose centrifuged at 35,000 rpm for 9 hours in an SW 39 Spinco head. The 1st fraction was from the bottom of the tube. O - - - - - - - - - O EDTA-treated HA; ●——————● Tween-ether-treated HA. The peak was 1.18 g/cm³ for the EDTA-HA and 1.21 g/cm³ for the Tween-ether-HA.

TABLE III. Effect of Cations of Hemagglutination by Rubella Virus.

Suspension medium for red cells	Titer of HA/ml
PBS*	1/320
NaCl†	<1/20
NaCl + 10^{-3} M $CaCl_2$	1/320
NaCl + 10^{-4} M $CaCl_2$	1/160
NaCl + 10^{-3} M $MgCl_2$	<1/20
NaCl + 10^{-4} M $MgCl_2$	<1/20

* PBS = phosphate buffered saline containing 10^{-3} M $CaCl_2$ and 10^{-3} M $MgCl_2$.
† .85% solution.

Sendai disease virus, were nevertheless agglutinated by rubella HA.

Tween-ether-prepared HA, on the other hand, was nonsedimentable under the conditions which pelleted EDTA-prepared HA, passed through a 100 mμ filter and was resistant to ether inactivation. Heat stability was slightly less than that of EDTA antigen (5 min at 56°C).

Effect of cations on hemagglutination. Calcium ions were essential to the hemagglutination of rubella virus (Table IV). While HA did not agglutinate 1-day-old chick cells in the presence of NaCl alone or of NaCl plus $MgCl_2$, the addition of $CaCl_2$ resulted in positive agglutination. The concentration of Ca^{++} in PBS was 10^{-3} M: HA titers equivalent to those in PBS were obtained by addition of 10^{-3} M $CaCl_2$ to isotonic saline.

In contrast to the attachment of HA to red blood cells, the attachment of HA to inhibitor took place in the presence of either Ca^{++} or Mg^{++}.

Effect of Tween-ether on EDTA-released HA. Tween-ether treatment of infected TCF resulted in the removal of serum inhibitor and release of HA, as described above. In addition, treatment of HA released from inhibitor by EDTA with Tween-ether resulted in a 2-fold increase in titer. The properties of the Tween-ether-treated EDTA antigen were similar to those recorded in Table III for Tween-ether antigen.

Elution of RV from red blood cells. RV could be eluted from hemagglutinated red blood cells by depriving the environment of divalent cations. EDTA-released or Tween-ether-released HA was added to a 25% red-blood cell suspension in PBS. After 2 hours adsorption at 4°C, the red cells were centrifuged. No HA was found in the supernatant after centrifugation. If the precipitated red cells were resuspended in PBS containing EDTA, and then recentrifuged, the HA reappeared in the supernatant (Table V). Resuspension in PBS without EDTA did not result in reappearance of HA. The virus could be attached and eluted to red cells at least 2 times.

TABLE IV. Characteristics of Rubella Hemagglutinins.

	HA released from tissue culture fluids by: EDTA	Tween-ether
Red cells agglutinated	One-day-old chicken most active; adult chicken and sheep also agglutinated; human, guinea pig, negative	Same
Optimal temperature	4°C better than 25°C, which is better than 37°C	"
Optimal pH	6.0–8.2	"
Filtration through 100 mμ millipore filter	No	Yes
Pelleted by 32,000 *g* for 150 min	Yes	No
RDE-treated red cells	Agglutinated	Agglutinated
Trypsin-treated red cells	"	"
Sendai virus-treated red cells	"	"
Stability in cold	>1 month at −20°C; >1 week 4°C	Same
Stability at 56°C	Unstable	Unstable
Ether treatment for 1 hr	Destroyed	Unaffected
Trypsin treatment of HA	Destroys	Destroys
Elution	Not spontaneous, but eluted by EDTA	Same
Calcium dependence	Yes	Yes
$HgCl_2$ and KIO_4 treatment	No effect	No effect

TABLE V. Effect of EDTA on Elution of Rubella Virus from One-Day-Old Chicken Red Cells.

Antigen/Treatment	EDTA-released antigen, HA/ml	Tween-ether-released antigen, HA/ml
Original HA	640	320
Supernatant after 2 hr adsorption to red cells	<1:2	<1:2
Supernatant after addition of .01 M EDTA	512	320
Supernatant after 2 cycles of adsorption-elution	640	320

After 2 such elutions the red cells remained susceptible to Sendai virus attachment, since addition of rubella virus-treated red cells to Sendai virus HA (titer 1/64) resulted in removal of the Sendai HA from the supernatant (residual titer 1/2). Agglutination of the red cells by Sendai virus was difficult to observe since the red cells to which rubella virus had been adsorbed and eluted showed spontaneous hemagglutination.

Size and characteristics of EDTA-released HA treated with Tween-ether. After EDTA-released HA was treated with Tween-ether, the antigen could not be sedimented by centrifugation at 100,000 *g* for 2½ hours and was filtrable through a 100 mμ filter. At the same time sucrose density gradient centrifugation showed that the HA had changed density. After 9 hours centrifugation, EDTA-released HA equilibrated at a density of 1.18 g/cm^3 while, after Tween-ether treatment, HA banded at a density of 1.21 g/cm^3 (Figure 2). Other centrifugations, performed under similar conditions, but for 48 hours, gave density values of 1.19 g/cm^3 for EDTA-HA, and 1.24 g/cm^3 for Tween-ether-HA.

Discussion. In addition to its size, RNA content, and ether sensitivity, the existence of a soluble complement-fixing antigen and of hemagglutinin suggests that rubella is a myxovirus. However, the characteristics of rubella hemagglutinin are not those of the myxoviruses. Rubella HA, in contrast to myxovirus, does not attach to mucopolysaccharide receptors, does not elute spontaneously from red cells, and is unstable at 56°C.

The attachment of rubella HA to serum inhibitor and to red cells is mediated by divalent cations. HA could be released from inhibitor by binding of cations. Addition of an excess of Ca^{++} or Mg^{++} prevented this release. On the other hand, Ca^{++} but not Mg^{++} was essential for agglutination of red cells by HA. The virus could be eluted by removing the cations, a fact which may be useful for virus concentration.

It is interesting that Krizanova and Sokol (7) found that influenza beta-inhibitor bound influenza virus only in the presence of Ca^{++}.

The nature of the serum inhibitor which is removed by kaolin or Tween-ether is the subject of another study(8), the results of which suggest that it is a beta-lipoprotein.

Treatment of infected tissue culture fluids with Tween-ether removed the serum inhibitor and broke down the intact virion. The smaller particle which resulted still gave hemagglutination.

The characteristics of hemagglutinin obtained from rubella virus are interesting with respect to viral taxonomy. Although rubella is generally classified as a myxovirus, removal of mucoprotein red cell receptors by RDE treatment did not prevent rubella hemagglutination. In fact, rubella HA resembles that of the arboviruses(9) in its greater activity with 1-day-old chick cells and in its failure to elute spontaneously. On the other hand, rubella HA is destroyed by lipid solvents, while arbovirus HA survives lipid extraction(9,10). Rubella HA shares some properties with measles HA, but the latter is stable at 56°C and is destroyed by potassium periodate treatment(11). These comparisons provide further evidence for the individuality of rubella virus and the necessity for a reclassification of myxoviruses(12).

Summary. The hemagglutinin (HA) of rubella virus can be released from serum inhibitor by binding divalent cations with EDTA. The HA is associated with the viral particle. HA can also be obtained by Tween-ether treatment of infected tissue culture fluids, in which case it is released from the intact whole virus. The attachment between red

cells and HA is dependent on divalent cations, and removal of the latter from the environment of agglutinated red cells results in elution of HA.

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Effect of Altered Plasma pCO_2 on Intracellular pH during Potassium Deficiency. (32561)

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Several laboratories have reported a decrease in skeletal muscle cell pH of K-deficient rats(1,2). But the nature of this increased cellular acidity is not known with certainty. Cooke *et al*(3) proposed that muscle cell pH decreases during K-deficiency because hydrogen ion (H^+) is transferred into cells from the extracellular compartment. This hypothesis is based on (i) the fact that K-deficiency in rats leads to a loss of muscle K that is two-thirds compensated by a gain of Na and (ii) the assumption that the difference represents a passage of H^+ into the cell. Recent experiments(2) together with earlier work reported from this laboratory (4,5) produced evidence that the reduced intracellular pH (pH_i) encountered in K-deficient rats is not related to the unequal replacement of muscle K loss by Na gain.

Another explanation for this reduced pH_i may be that CO_2 diffuses from plasma into skeletal muscle during potassium deficiency. This was suggested by Miller *et al*(6) based on the observation that plasma pCO_2 is elevated during K-deficiency. If diffusion of CO_2 into the low-K cell is the major cause of its acidity, the pH_i of control and low-K muscle should be the same at identical pressures of CO_2.

To determine the degree of cellular acidity resulting from our K-depletion regimen, we altered the plasma pCO_2 of normal and K-deficient rats with a respirator and then measured pH_i by 5,5-dimethyl-2,4-oxazolidinedione (DMO) distribution. The data enabled a comparison of normal and low-K muscle pH_i at CO_2 pressures ranging from 20 to 90 mm Hg to be made.

Methods. Young adult male rats (Wistar strain) weighing about 270 g were given the control or the low-K diet used previously(2). The low-K diet contained normal amounts of Na and Cl. The rats were housed in individual cages and given food and water *ad libitum*. Animals receiving control diet were maintained on this diet for 8 days; those to be depleted of K were given low-K diet for 35 days.

About 3½ hours before termination of an experiment, 60 mg of DMO/kg of body wt was injected intraperitoneally. Seventy-five minutes later, the rat was placed under sodium pentobarbital (50 mg ip/kg body wt) anesthesia. The trachea was exposed and con-