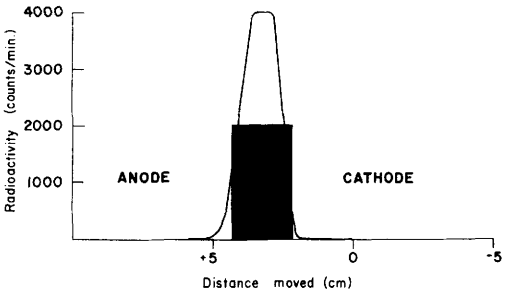

FIG. 1. Typical radiochromatogram scan of H^3 heparin electrophoretic pattern. Location of metachromasia peak obtained with toluidine blue shown as darkened bar.

(9) reported on a catalytic exchange method using tritium oxide and transition metals as catalysts. We have found that palladium on carbon catalyzes the tritium exchange reaction to the extent that specific activities of some compounds are high enough to permit their use as tracers in biological experiments. Tritiated heparin is an excellent example of this type of reaction, the final product having 18.2×10^6 dpm/mg, which allows for accurate measurement of submicrogram quanities.

Radioactive heparin prepared by this method has been used by Kramer(10) in studies on the stability of the GBH surfaces. The remarkable stability of this compound was shown by dissolution in saline at 24°C, in a tightly sealed container. *In vacuo* distillation of the solution 2-1/2 months thereafter demonstrated that only 5.2% of the contained activity was present as ^3H_2O; the remaining material was still precipitable with protamine sulfate, indication that 90% of the tritium was still organically bound.

The 50% yield of tritiated heparin indicates that the tritiation process brings about considerable degradation. The elimination of these breakdown products is apparent in the first precipitation with CPC, where the bulk of the radioactivity remains in the supernatant. It is possible that a single purification would suffice since the radioactivity in the supernatant following the second precipitation was minimal.

The availability of tritiated heparin should lead to further investigations on the metabolic fate of this interesting biopolymer. It is expected, but has not been demonstrated, that the tritium label is more stable than the S^{35} label on heparin.

1. Jorpes, J. E., Heparin, Oxford, London, 1946.
2. Eiber, H. R., Danishefsky, I., J. Biol. Chem., 1957, v226, 721.
3. ———, Nature, 1957, v180, 1359.
4. Eiber, H. R., Danishefsky, I., Borrelli, F. J., Proc. Soc. Exp. Biol. and Med., 1958, v98, 672.
5. Levy, L., Petracek, F. J., ibid., 1962, v109, 901.
6. Gott, V. L., Whiffen, J. D., Dutton, R. G., Science, 1963, v142, 1297.
7. Barlow, G. H., Biochem. et Biophys. Acta, 1964, v83, 120.
8. Yavorsky, F. M., Gorini, E., J. Am. Chem. Soc., 1962, v84, 1071.
9. Garnett, J. L., Nucleonics, 1962, v20, 86.
10. Kramer, R., to be published.

Received September 8, 1966. P.S.E.B.M., 1966, v123.

Production of Rubella Complement Fixing Antigen in BHK-21 Cells.* (31617)

KLAUS SCHELL, K. T. WONG, H. C. TURNER, AND R. J. HUEBNER
Laboratory of Infectious Diseases, NIAID, National Institutes of Health, and Microbiological Associates, Inc., Bethesda, Md.

Serological diagnosis of rubella infection has been complicated in the past by the lack of a simple, quick, and accurate method of measuring the development of rubella antibodies. The generally applicable technique of fresh cell sedimentation (cell packing) for concentrating viral complement fixing (CF) antigens demonstrated recently was used for the concentration of rubella CF antigen (1,2).

Sever *et al*(2) have shown the usefulness of the complement fixation test for the demonstration of rubella antibody. In this publi-

* Part of this work was supported by USPHS Contract PH 43-62-200.

cation we confirm earlier observations that infection of BHK-21 cells(3) provides an excellent way to produce rubella CF antigen and we describe refinements leading to improved antigen yields.

Materials and methods. Tissue culture. BHK-21 cells (serial baby hamster kidney) were obtained from Dr. R. Tennant† and propagated in our laboratory employing the following procedure: Monolayers were washed twice with 0.25% viokase at room temperature to dislodge the cells. Cells were suspended in Eagle's Minimum Essential Medium(4) in Hanks' balanced salt solution (HMEM) containing 10% fetal calf serum. The cells from one flask were distributed over 4, and incubated at 37°C. Under these conditions confluent monolayers were obtained in 2-3 days. Cells were maintained at 34°C on the above medium with 3% fetal calf serum. For virus propagation, fetal calf serum was replaced by 10% agamma calf serum.‡

Primary African green monkey kidney (AGMK) cells, RK-13 and MA-111 (serial rabbit kidney cell lines), MA-104 (serial Rhesus monkey kidney line), MA-134 and BSC-1 (serial AGMK lines), and primary hamster kidney and human amnion cells were obtained from Microbiological Associates, Inc. All were maintained at 34°C on Eagle's Minimum Essential Medium(4) with Earle's balanced salt solution (EMEM) containing 3% newborn calf serum. All media contained 100 units penicillin and 100 γ streptomycin per ml.

Virus. Rubella virus, strain 33-BRSTI and Echo virus 11, strain Gregory, were obtained from Dr. R. R. Rafajko.§ The rubella virus originally had been obtained from Dr. Gilbert M. Schiff. It was produced in BHK-21. Rubella multiplicities of infection (MOI) were estimated on the basis of average BHK-21 cell counts, *e.g.*, $10^{6.8-7.1}$ cells per C200 flask (Milk dilution bottle: 50 cm^2 monolayer) and $10^{7.1-7.4}$ cells per C32 flask (32 ounce Prescription bottle: 120 cm^2 monolayer). Echo virus 11 was grown in AGMK cells.

Infectivity titrations of rubella virus were carried out in AGMK cells employing as indicator system interference with Echo 11 virus(5). Monolayered roller tubes were inoculated with 10-fold dilutions of the virus and then challenged 7 days later with 10-100 ID50 of Echo 11. Titers were read 3 days after challenge as the highest dilution interfering with the Echo virus CPE and calculated according to Reed and Muench(6).

Antigen preparation. Cell cultures were harvested as cell packs in a manner described earlier(1): Infected cells were scraped into the supernatant medium, centrifuged for 10 minutes at 1000 rpm in an International PR2 and resuspended in 1/20th of the supernate (20×). This 20× cell concentrate was equivalent to a 3-4% cell suspension by packed volume depending on quality of monolayer. Antigen was released from the cells by freezing and thawing 3 times or by homogenization for one minute at maximum speed in a Virtis "45" homogenized. Only an occasional preparation showed traces of anticomplementary activity at a dilution of 1:2. Non-infected cell preparations were used as controls.

Antisera. Paired acute and convalescent sera from rubella patients were provided by Dr. J. L. Sever.

Complement fixation tests were carried out in the serology laboratory of the Laboratory of Infectious Diseases, NIAID, National Institutes of Health, employing the microtiter‖ system as described by Sever(7). Titers were recorded as the reciprocal of the highest dilution showing 3+ or 4+ fixation (within a range of 0 to 4+) of 1.8 full units of guinea pig complement.

Experiments and results. The antigen contents in "packed cell" preparations of BHK-21 cultures infected with rubella virus were determined in tests with known positive convalescent sera from rubella patients. The development of antibody in such convalescent sera as compared to acute antibody-free sera from the same patients served as evidence

† Present address: Albert Einstein Med. Center, Dept. of Microbiology, Research Laboratories, Philadelphia, Pa.

‡ Hyland Laboratories, Los Angeles, Calif.

§ Microbiological Associates, Inc., Bethesda, Md.

‖ Cooke Engineering, Alexandria, Va.

TABLE I. Detection of Antibody Rises in Paired Sera from Rubella Patients Using Several Lots of Antigen Made in BHK-21 Cells.

Antigen* lot #	Serum source	Acute serum titer	Convalescent serum titer
1	Patient #1 (#99) #265	<5	5–10
	Sellers, C. (12/11) 4/29	<5	10–20
2	Mallory (#22368) #24804	<5	>20
	Patient #1 (#99) #265	<5	10
	Sellers, C. (12/11) 4/29	<5	20
3	Mallory (#22368) #24804	<5	>20
	Patient #1 (#99) #265	<5	10
	Sellers, C. (12/11) 4/29	<5	20

* Four units of antigen were used for detection of rubella antibodies.

that the antigens obtained were indeed rubella CF antigens (Table I).

Neva and Weller(8) demonstrated that the rubella interfering mechanism was, in part at least, due to interferon production. We attempted, therefore, during the initial virus growth period, to remove interferon by daily medium changes and washes with viokase (0.25%). The procedures used were as follows: C200 flasks (square bottles with 50 cm^2 surface area for cell growth) with BHK-21 monolayers were inoculated with aliquots of rubella virus. One of two flasks per replicate preparation was washed with viokase once a day during the first 3 days, the second flask being held as unwashed control. Viokase washing was done at room temperature with 0.25% viokase flushed generously over the monolayer keeping time of exposure to a minimum.

The results shown in Table II indicated that this procedure provided increased antigen yields. Subsequent experiments, however, failed to confirm the need for viokase, but yields decreased when intervals between washings were lengthened to 2 or more days or when washing was omitted altogether.

These experiments also suggested that larger amounts of serum in the medium resulted in higher antigen yields than when smaller amounts were used. Subsequent replicate tests confirmed this observation (Table III).

The effects of different incubation temperatures and input multiplicities on complement fixing antigen yields were investigated. The total antigen yields achieved were very similar when the cultures were maintained at temperature ranges of 30° to 37°C; however, the antigen yields were low at 27°C, and there was no antigen produced at 40°C. Decreasing doses of virus from a multiplicity of infection of 1/3 to 1/3000 did not affect antigen yields but delayed the appearance of peak antigen titers.

We also compared antigen titers of cell packs resuspended in varying volumes of supernate: Eighteen C32 flasks were inoculated with aliquots of rubella virus (MOI of 1/10) and harvested 5 days later using increasing volumes of supernatant medium for resuspension of the cell packs. Cell pack concentrations and antigen titers were directly proportional, indicating that most of the

TABLE II. Washing of Cell Monolayer and the Development of Complement Fixing Antigens.

	Replicate preparations			
	1		2	
Harvest (day p.i.)	Washed	Unwashed	Washed	Unwashed
Exp S367 (MOI: 1/50)*				
3	0†	0	0	0
4	16	0	4	0
5	8	0	8	2
6	2	0	8	2
7	4	2	2	2
Exp S484 (MOI: 1/3)*				
5	>32	8	32	8
Exp S497 (MOI: 1/3)*				
5	>32	32	>32	16
6	>32	8	16	16

* Multiplicity of infection: No. of ID50/No. of cells.

† Complement fixation (reciprocal of titer).

p.i. = post inoculation.

Note: Maintenance media for experiments S484 and S497 contained 10% agamma calf serum instead of 3% (S367).

TABLE III. Influence of Serum Concentration on CF Antigen Production.

Harvest (days p.i.)	Proportion of agamma calf serum in maintenance medium 3%	5%	10%
Exp S428 (MOI: 1/5)*			
3	0†		0
4	2		8
5	4		16
6	8		>16
7	8		16
8	4		8
Exp S484 (MOI: 1/3)*			
5	8	16	>32

* Multiplicity of infection: No. of ID50/No. of cells.

† Complement fixation (reciprocal of titer).

p.i. = post inoculation.

complement fixing antigen was retained inside the cells until harvesting.

Selecting a time for harvesting cells containing an optimal amount of antigen was facilitated by observations of changes in the pH of the media and the development of cytopathic effects. Rubella infection of BHK-21 cells was accompanied by acidification, indicating an increased rate of cell metabolism. Acidification below pH 7 as a rule preceded CF antigen peaks by one or two days. This held true even when cultures were incubated at various temperatures or when they had been inoculated with various multiplicities of infection (1/3 to 1/3000). The yields of antigen were not altered by the use of media containing different buffers or buffer concentrations; *e.g.*, HMEM, EMEM, Medium 199 (9), Medium L-15 (Leibovitz) (10). It should be noted that in these tests all the media contained 10% serum which might have buffered minor pH differences. Cytopathic effects were observed in BHK-21 cell cultures inoculated with rubella virus, usually before the complement fixing antigen content reached its peak titer. Although CPE was generalized when high multiplicities of infection were used, focal lesions were observed with less virus. Foci consisted of granulation and rounded cells which became more numerous as incubation time increased. The rounded cells in the foci eventually detached from the glass resulting in the appearance of holes in the cell sheet. This CPE was particularly pronounced when the cultures were washed frequently Convalescent sera from rubella patients neutralized the cytopathic effects.

Discussion and summary. The assays for antibodies to rubella virus are time consuming and complicated by the lack of simple and accurate indicator systems. The recent development of rubella CF antigen in RK-13 and AGMK cells(2) as well as in BHK-21 cells (1) made it possible to measure complement fixing antibodies quickly and accurately in a well standardized system(7). However, whereas RK-13 and AGMK cells had to be concentrated over 200-fold (30% cell suspension) to yield antigen titers of 1/8(2), BHK-21 cells required only 20-fold concentration (3-4% cell suspension) to produce similar titers without anticomplementary complications.

The production procedure described here resulted in improved antigen yields of consistently high titers sufficient to carry out reproducible, highly satisfactory tests for complement fixing antibodies to rubella. Peak antigen titers were preceded by the appearance of cytopathic effect and acidification of the maintenance medium. The antigen produced by this procedure reduced the costs of production sufficiently to make the complement fixation test a diagnostic tool feasible for use in epidemiological studies of rubella infections and for evaluation of immunogenic properties of future rubella vaccines.

We wish to thank Mr. M. M. Vincent who provided various experimental cell lines, and Dr. J. L. Sever who provided rubella antisera.

1. Schell, Klaus, Huebner, R. J., Turner, H. C., Fed. Proc., 1965, v24, 249.

2. Sever, J. L., Huebner, R. J., Castellano, G. A., Sarma, P. S., Fabiyi, A., Schiff, G. M., Susumano, C. L., Science, 1965, v148, 385.

3. MacPherson, I., Stoker, M., Virology, 1962, v16, 147.

4. Eagle, H., Science, 1959, v130, 432.

5. Parkman, P. D., Buescher, E. L., Artenstein, M. S., Proc. Soc. Exp. Biol. and Med., 1962, v111, 225.

6. Reed, L. J., Muench, H., Am. J. Hyg., 1938, v27, 493.

7. Sever, J. L., J. Immunol., 1962, v88, 320.

8. Neva, F., Weller, T. H., ibid., 1964, v93, 466.

9. Morgan, J. F., Morton, H. J., Parker, R. C.,

Proc. Soc. Exp. Biol. and Med., 1950, v73, as modified by Salk, J. E., Youngner, J. S., Ward, E. N., Am. J. Hyg., 1954, v60, 2.

10. Leibovitz, A., ibid., 1963, v78, 173.

11. Issacs, A., Burke, D. C., Brit. Med. Bull., 1959, v15, 185.

12. De Maeyer, E., Enders, J. F., Proc. Soc. Exp. Biol. and Med., 1961, v107, 573.

Received September 9, 1966. P.S.E.B.M., 1966, v123.

Effect of Magnesium Deficiency on Location of the Intestinal Absorption of Magnesium in Rats. (31618)

JERRY G. CHUTKOW* (Introduced by L. O. Jacobson)

Argonne Cancer Research Hospital† and Department of Medicine, University of Chicago, Chicago, Ill.

The absorption of magnesium is increased in young rats fed a diet low in magnesium(1). In the following studies, Mg^{28} was used to determine whether this alteration is primarily associated with a local or general enhancement in the uptake of magnesium from the alimentary tract.

Methods. Non-fasting male albino rats (Sprague-Dawley strain) weighing 90 to 110 g at the beginning of the experiments were housed in individual metabolism cages that provided separation of the urine and the feces. The animals were fed either a low magnesium or a magnesium supplemented (control) diet (1) and distilled water *ad libitum*.

Magnesium-28 as $Mg^{28}Cl_2$ with a specific activity of 16 to 20 μC of Mg^{28}-Al^{28} per mg of stable magnesium at the time of administration was obtained from Brookhaven National Laboratory. Detailed descriptions of the equipment and methods have been presented elsewhere(1,2). In all the experiments, each animal received approximately 2 μC of Mg^{28} in 0.5 ml of 5% dextrose in water containing an additional 3 mg of magnesium as $MgCl_2 \cdot 6H_2O$. The extra magnesium was added to compensate, in part, for the much greater dilution of specific activity of the Mg^{28} which would otherwise occur in the bowel of the control rats due to ingested dietary magnesium. Except for net plasma counts lower that 100 cpm, the per cent probable error of the net counting rate was never greater than 3% and usually 2% or less.

Unless otherwise noted, the animals were anesthetized with ether during the administration of Mg^{28} and operative procedures. All blood samples were obtained by aortic exsanguination using heparin as an anticoagulant. Plasma magnesium levels were determined in duplicate on a Beckman B spectrophotometer by the titan yellow method (3,4).

Except for the multiple comparison analysis of Scheffe(5), statistical methods were taken from Snedecor(6).

Experiment 1. Absorption of Mg^{28} after direct injection through the bowel wall into the gastric or colonic lumen. Two studies of identical design were performed on a total of 51 animals, and the results were combined.

The rats were divided into 4 groups and placed on the magnesium supplemented diet. After an initial period of adjustment, 2 of the groups were transferred to the low magnesium diet. Four days later, 0.5 ml of the solution containing the Mg^{28} was injected directly into either the gastric or cecal lumen of each animal using a No. 28 needle through an abdominal incision. (Preliminary studies in which carmine red dye was used as an indicator showed that there was no loss through the puncture site.) During injection into the cecum, the ileum was gently compressed at the ileocecal junction to prevent any initial large reflux of the isotope. The abdomen was closed with sutures and metal clips, and feces were collected over the next 44 hours.

* USPHS Special Fellow in Neurology.

† Operated by University of Chicago for U.S. Atomic Energy Commission.