(21). It is also possible that the effect of nucleic acid inhibitors is due to inhibition of interferon production. In the RV-BHK21 cell system, however, this is unlikely, as no interference appears to be induced against vesicular stomatitis and Newcastle disease viruses (22). The enhancement of RV by ACTD and FUdR might also be due in part to moderation of the overall cell metabolism in cell cultures and resultant better maintenance.

Summary. The excellent growth of rubella virus in BHK21 cells is further enhanced by a number of substances. DEAE-dextran increased the plaquing efficiency, yield of virus, and cytopathic effect of the virus, particularly when present during virus adsorption. The incorporation of semicarbazide into maintenance media improved plaquing efficiency and cytopathic effect, but not virus yield. Other substances such as actinomycin and 5 fluoro-2'deoxyuridine, when present in low dosages in the maintenance media of infected cells, increased virus yield, cytopathic effect, and plaquing efficiency.

1. Vaheri, A., Sedwick, W. D., Plotkin, S. A., Proc. Soc. Exp. Biol. & Med., 1967, v125, 1092.

2. Vaheri, A., Pagano, J. S., Virology, 1965, v27, 434.

3. Pagano, J. S., Vaheri, A., Arch. ges. Virusforsch., 1965, v17, 456.

4. Lwoff, A., Biochem. J., 1965, v96, 289.

5. Grossberg, S. E., Lwoff, A., Lwoff, M., Feder. Proc., 1966, v25, 492.

6. Vaheri, A., Sedwick, W. D., Plotkin, S. A., Maes, R., Virology, 1965, v27, 239.

7. Vaheri, A., Cristofalo, V. J., Arch. ges. Virusforsch., in press.

8. Vaheri, A., Acta Path. Microbiol. Scand., Suppl., 1964, v171, 1.

9. Maes, R., Vaheri, A., Sedwick, W. D., Plotkin, S. A., Nature, 1966, v210, 384.

10. Hannoun, C., Fernandes, M. V., Maciera-Coelho, A., Proc. Soc. Exp. Biol. & Med., 1965, v119, 153.

11. Allison, A. C., Sandelin, K., J. Exp. Med., 1963, v117, 879.

12. Polet, H., Exp. Cell Res., 1966, v41, 316.

13. Maes, R., Wiktor, T., Campbell, J., Koprowski, H., Kaplan, M., The Molecular Biology of Viruses, Colter, J. S., ed., Academic Press, New York, 1967, pp. 449-462.

14. Takemoto, K. K., Liebhaber, H., Virology, 1961, v14, 456.

15. Smull, C. E., Ludwig, E. J., J. Bact., 1962, v84, 1035.

16. Amos, H., Kearns, K. E., Exp. Cell Res., 1963, v32, 14.

17. Ryser, H. J. P., Hancock, R., Science, 1965, v150, 501.

18. Maes, R., Sedwick, W. D., Vaheri, A., Biochim. Biophys. Acta., in press, 1967.

19. Waterson, A. P., Arch. ges. Virusforsch., 1965, v15, 275.

20. Maassab, H. F., Cochran, K. W., Proc. Soc. Exp. Biol. & Med., 1964, v117, 410.

21. Barry, R. D., Cellular Biology of Myxovirus Infections, Wolstenholme, G. E. W., Knight, J., eds., Ciba Foundation Symposium, J. & A. Churchill, Ltd., 1964, pp. 51-71.

22. Sedwick, W. D., Unpublished observations, 1966.

Received March 29, 1967. P.S.E.B.M., 1967, v125.

Growth of Rubella Virus in BHK21 Cells. III. Production of Complement-Fixing Antigens.* (32285)

T. FURUKAWA, A. VAHERI,† AND S. A. PLOTKIN‡
(Introduced by D. Kritchevsky)
Wistar Institute of Anatomy and Biology, Philadelphia, Pa.

Complement-fixing (CF) antigens for rubella virus have been reported in a number of cell systems: Sever *et al*(1) reported the development of a cell-associated antigen in infected RK_{13} and African green monkey kidney cells. Soluble CF antigens were found by Schmidt and Lennette(2) in concentrated

* This investigation was supported in part by USPHS Research Grant AI-01799 from Nat Inst. of Allergy & Infect. Dis. and the Joseph P. Kennedy, Jr. Foundation.

† Present address: Virus Laboratory, Orion OY, Helsinki 51, Finland.

‡ Joseph P. Kennedy, Jr., Foundation scholar.

supernatants from RK_{13} cells late in infection, and by Veronelli *et al*(3) in concentrated fluids harvested from LLC-MK_2 cultures. Chronically infected LLC-MK_2 cells—the RAL carrier line — produce rubella CF antigen(4). The production of high titered rubella CF antigens in BHK21 cells has been reported previously by Schell *et al*(5). This paper gives details of production of CF antigen in BHK21 and the results of analysis of the antigens produced.

Materials and methods. The BHK21 cell lines, the RA 27/3 rubella virus strain, and the methods used for cell culture and virus assay are described in the first paper in this series(6).

Preparation of cell pack antigens. The cell monolayer was scraped from the glass with a rubber spatula and centrifuged at 1000 rpm for 10 minutes in an International PR 2 Centrifuge. The sedimented cells were suspended in the supernatant fluid to give a 10% cell concentration; this suspension was frozen and thawed 3 times in dry ice and alcohol, the final material constituting the antigen.

Preparation of fluid antigens. Supernatant fluids were clarified by centrifugation at 2000 rpm for 20 minutes. The clarified fluids were concentrated a hundredfold by dialysis against polyethylene glycol (Carbowax 20 M) overnight at 4°C.

Purification of virus and sucrose density gradient centrifugation. These procedures are described elsewhere(6). The only difference was that in the present work the gradients were centrifuged for 9 hours.

Complement-fixation test. CF tests were done in the microtiter system described by Sever(7). Antigens and control antigens were diluted serially in veronal-buffered saline. Following addition of 0.025 ml of complement (containing 2 exact units), the plates were incubated overnight at 4°C, after which the hemolytic system (0.05 ml of 1% sensitized sheep red blood cells) was added. After this mixture was incubated at 37°C for 1 hour, a reading was made. The sera employed for CF testing, *i.e.,* rubella convalescent sera and sera from rubella syndrome infants, had titers of 1:32.

Virus assay. Plaque assays were performed in BHK21/WI-2 cells as described previously(6).

TABLE I. Rubella CF Antigen Development in BHK21/13S Suspension Culture.

Hours after infection	Cell associated CF antigen*	Supernatant CF antigen (conc. 100×)	Infectivity† (fluid phase)
18	No activity	<1:2	$10^{6.4}$
42	1:2	<1:2	$10^{6.9}$
66	1:4	1:8	$10^{6.8}$
90	1:4	1:16	$10^{6.9}$
138	1:16	1:8	$10^{6.6}$

Input of multiplicity virus/cell = 5/1.
* 10% suspension.
† pfu/ml: titrated in BHK21 WI-2 culture.

Results. Production of CF antigen. The BHK21/13S cell line infected with rubella virus in suspension or in monolayer, was tested for the production of rubella CF antigen. In experiments with monolayer cultures, the maximum titer of supernatant virus was generally reached at 5 days after infection. Cell-associated CF antigen reached a peak at 3 days after infection. CF antigen was also demonstrated in the supernatant fluids after a hundredfold concentration. Results obtained in monolayer culture, however, were not as satisfactory as those obtained in suspension.

The results of an experiment performed with suspension cultures are given in Table I. A CF titer of 1/16 was produced in a 10% cell pack at 5 days. A similar titer was obtained in concentrated supernatant fluid at 4 days. From the second to the fourth day the virus yield in the supernatant was almost $10^{7.0}$ PFU/ml.

13S cells in suspension culture, chronically infected with rubella virus, produced CF antigen for at least 23 days, at which point the culture became contaminated (Table II). Virus yields in the supernatant fluid also were continuously high.

The nature of CF antigen. Elsewhere(6) we report the partial purification of rubella virus. To determine whether CF antigen was associated with purified virus, partly purified virus was placed on a sucrose density gradient. CF activity was found only in the fractions with the highest infectivity (Table III).

A partial purification was performed to determine the nature of the CF antigen detectable in the tissue culture supernatant

TABLE II. Rubella CF Antigen in Chronically Infected BHK21/13S Suspension Culture.

Time after infection	Cell associated antigen*	Infectivity (fluid)
9 hr	<1:4	$10^{3.3}$
20 "	<1:4	$10^{3.9}$
33 "	<1:4	$10^{4.8}$
44 "	<1:4	$10^{6.3}$
56 "	1:4	$10^{6.4}$
72 "	1:8	$10^{6.5}$
95 "	1:16	$10^{6.5}$
2 days after split	1:8	$10^{6.5}$
4 days	1:4	$10^{6.7}$
6 "	1:4	$10^{6.5}$
8 "	1:8	$10^{6.7}$
14 "	1:8	$10^{6.8}$
23 "	1:4	$10^{6.9}$

* 10% suspension.

fluids; CF antigen was tested at each step. After centrifugation sufficient to precipitate virus, although part of the antigen was sedimented, part remained in the supernatant (Fig. 1). Tween-ether treatment performed according to the method of Norrby(8) did not enhance the titer of soluble antigen.

Supernatant tissue culture fluid harvested 4 days after infection of BHK21/13S cells in monolayer was centrifuged on a sucrose density gradient. The peak of infectivity lay between densities 1.17 and 1.21 (Fig. 2). CF activity was found both at this point and at a lower density (1.06-1.08) where there was little infective virus.

When the supernatant fluid was harvested at 7 days after infection while the cells were showing pathologic effects and virus infectivity had fallen off, CF activity was localized in the lower density fractions of the gradient, with little if any activity related to the fractions containing whole virus (Fig. 3).

TABLE III. Rubella CF Antigen in the Fractions of a Sucrose Density Gradient of Partially Purified Virus.

No. fraction	CF activity (1:10)	Infectivity	d_4^{20}
1	—	$<10^{5.6}$	1.245
2	—	$<10^{5.6}$	
3	—	$<10^{5.6}$	1.227
4	—	$10^{6.8}$	
5	+	$10^{7.8}$	1.196
6	—	$10^{7.1}$	
7	—	$<10^{5.6}$	1.159
8	—	$<10^{5.6}$	
9	—	$10^{6.1}$	1.121
10	—	$<10^{5.6}$	
11	—	$<10^{5.6}$	1.084
12	—	$<10^{5.6}$	
13	—	$<10^{5.6}$	1.056
14	—	$<10^{5.6}$	
15	—	$<10^{5.6}$	1.033
16	—	$<10^{5.6}$	
17	—	$<10^{5.6}$	1.029

It was possible to estimate the amount of infectious virus particles necessary to produce one unit of CF activity. The results of testing 4 different preparations of purified virus are given in Table IV. One unit of CF activity

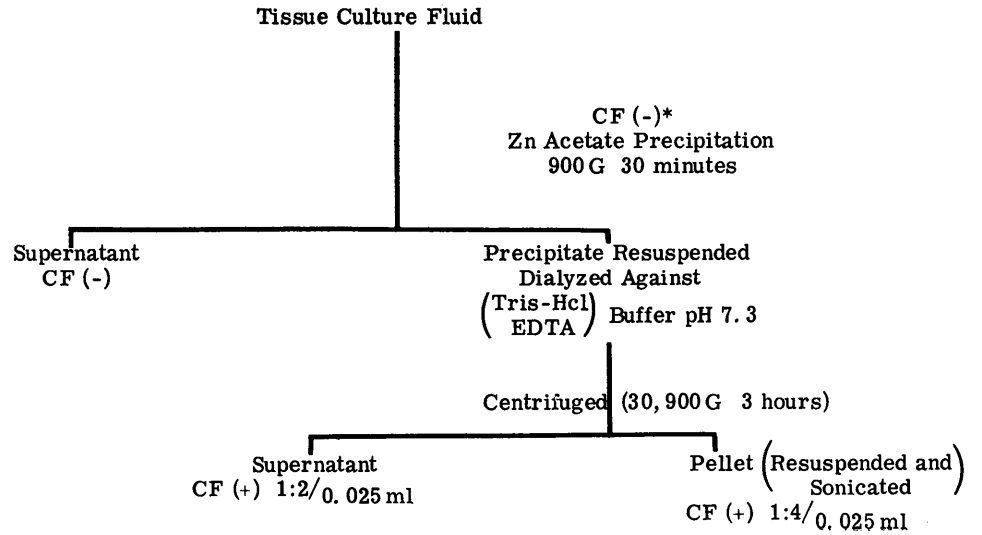
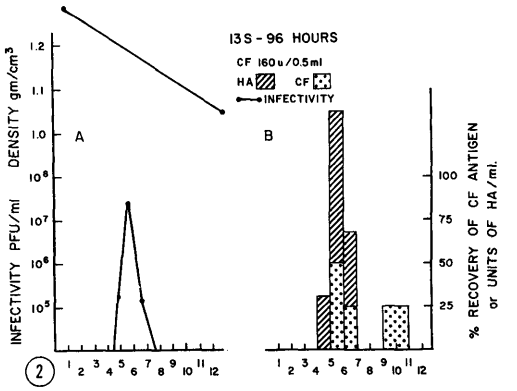


FIG. 1. Rubella CF activity during partial purification of virus.

corresponded to about 100,000 PFU.

Stewart *et al*(9) have recently described a rubella hemagglutinating antigen. We tested the sucrose density gradient fractions for hemagglutinating antigen, using one-day-old chick cells as described by the original authors. The results (Figs. 2 and 3) show that HA antigen is associated with the peaks of virus infectivity.

Discussion. We have demonstrated that rubella CF antigen is produced in large amounts in infected BHK21/13S cells and that such antigen can be harvested repeatedly from chronically infected cells in suspension. The CF antigen produced in this system is of two types: one is associated with the virus particle and one is soluble. The two types could be separated on sucrose density gradients.



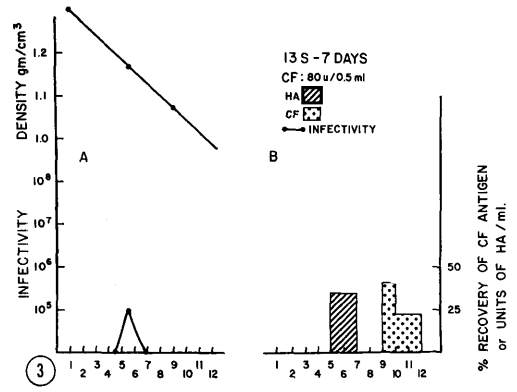


FIG. 2. Sucrose density gradient centrifugation of supernatant tissue culture fluid obtained from BH K21/13S cells, 96 hr after infection. The density and the infectivity are shown in part A; the complement-fixing and hemagglutinating activity are shown in part B. The density of each fraction was measured by refractometer.

FIG. 3. Sucrose density gradient centrifugation of supernatant tissue culture fluid obtained from BHK21/13S cells, 7 days after infection. The density and the infectivity are shown in part A; the complement-fixing and hemagglutinating activity are shown in part B. The density of each fraction was measured by refactometer.

TABLE IV. Relationship of Rubella Virus Infectivity to Content of CF Antigen.

Exp No.	pfu/ml	cfu/ml (units)	pfu/cfu
1	$10^{7.8}$	400	5.2
2	$10^{7.6}$	400	5.0
3	$10^{7.4}$	40	5.8
4	$10^{7.1}$	160	4.9

Early in the cycle of virus production in BHK21, when large amounts of infective virus were produced, the CF antigen obtained from the tissue culture fluids was largely sedimentable, and was demonstrated in sucrose gradient fractions having a density of 1.18-1.21 g/l. Furthermore, partially purified virus from supernatant fluid gave a CF reaction in direct proportion to infectivity.

In contrast, in cultures no longer producing much infectious virus, a soluble antigen was found predominantly in the sucrose fractions having a density of 1.06-1.08 g/l. Thus the soluble CF antigen of rubella resembles that of other myxoviruses with regard to noninfectivity and small particle size.

These results confirm those of Schell and Wong(5), who showed the late appearance of soluble antigen in rubella-infected BHK21 cultures, and Schmidt, Lennette and others (2,10), who separated the two antigens on Sephadex G-200 gel.

In addition we have shown that the recently discovered hemagglutinating antigen also was associated with the whole virus particle.

Summary. The production of rubella CF antigen was studied in a cloned line of BHK21 cells cultured in monolayer or in suspension. Two antigens were demonstrated: one present in purified virus preparation and associated with the virus particle, and the second soluble and tending to appear late in the course of infection of BHK21 tissue culture.

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

2. Schmidt, N. J., Lennette, E. H., Proc. Soc. Exp. Biol. & Med., 1966, v121, 243.
3. Veronelli, J. A., Eckert, E. A., ibid., 1966, v121, 1223.
4. Stern, H., Nature, 1965, v208, 200.
5. Schell, K., Wong, K. T., ibid., 1966, v212, 621.
6. Vaheri, A., Sedwick, W. D., Plotkin, S. A., Proc. Soc. Exp. Biol. & Med., 1967, v125, 1086.
7. Sever, J. L., Ley, A. C., Wolman, F., Caplan, B. M., Crockett, P. W., Turner, H. C., Am. J. Clin. Path., 1964, v41, 167.
8. Norrby, E., Proc. Soc. Exp. Biol. & Med., 1962, v111, 814.
9. Stewart, G. L., Parkman, P. D., Hopps, H. E., Douglas, D. D., Hamilton, J. P., Meyer, H. M., Jr., New Engl. J. Med., v276, 554.
10. Schmidt, N. J., Lennette, E. H., Gee, P. S., Proc. Soc. Exp. Biol. & Med., 1966, v123, 758.

Received March 29, 1967. P.S.E.B.M., 1967, v125.

Inhibition of Ovarian Ascorbic Acid Depletion with Ovine Luteinizing Hormone Antiserum.* (32286)

P. C. HOPPE, S. K. QUADRI,† AND H. G. SPIES‡
(Introduced by C. H. Sawyer)
Department of Animal Husbandry, Kansas State University, Manhattan, Kansas

Moudgal and Li(1) reported that rabbit antiserum prepared against ovine luteinizing hormone (LH) neutralized endogenous and exogeneous LH in the rat. This antiserum also neutralized LH preparations of pig, whale, and human pituitaries or pregnant mare's serum (PMS), but had no influence on chicken LH or human chorionic gonadotropin (HCG) when tested by ventral prostate weight in hypophysectomized rats. Bourdal (2) presented evidence that rabbit antiserum to ovine LH suppressed increases in ovarian and uterine weights of immature intact female rats, and subsequently, Bourdal and Li(3) blocked vaginal cycles in adult rats. Rennels(4) has reported that antibodies against LH prevented ovarian ascorbic acid depletion in immature pseudopregnant rats when injected ip 30 minutes prior to iv LH or when the antiserum and LH were incubated together *in vitro* prior to injection. He observed that sc injections of antiserum were ineffective, and hypothesized that the ineffectiveness was due to the rate of absorption of the antibody.

The present study was conducted to determine the influence of an iv injection of rabbit ovine-LH-antiserum on ovarian ascorbic acid depletion (OAAD) by LH in immature pseudopregnant rats. It was of interest, also, to determine if the ability of the antiserum to inactivate standard amounts of LH *in vivo* would correspond with estimates of the antibody predicted by a precipitin test.

Materials and methods. Adult male rabbits were immunized against ovine LH-S8 by 4 weekly 1.5 mg sc injections of LH dissolved in 1.5 cc of saline homogenized in an equal volume of Freund's complete adjuvant (Difco, Detroit, Mich). Two 2 mg booster injections of LH were given at 2-week intervals subsequent to the fourth 1.5 mg injection. After an additional time lapse of 12 weeks, a third 2 mg booster injection was administered followed by a final 2 mg injection 2 weeks later. Four days following the third and fourth booster injections 25-50 cc of blood was withdrawn (ear vein) from each rabbit. Adjuvant serum to Freund's complete adjuvant was prepared in the same manner in 2 male rabbits. Control serum was obtained from normal male rabbits. The sera within each treatment were pooled, and the globulins were concentrated to one-third of the original

* Supported in part by NIH Grant HD-00392. Contribution No. 347, Dept. of Animal Husbandry, Kansas Agr. Exp. Station, Manhattan, Kan.

† Present address: Dept. of Microbiology and Public Health, Michigan State Univ., East Lansing, Mich.

‡ Present address: Dept. of Anatomy, U.C.L.A. School of Medicine, Los Angeles.