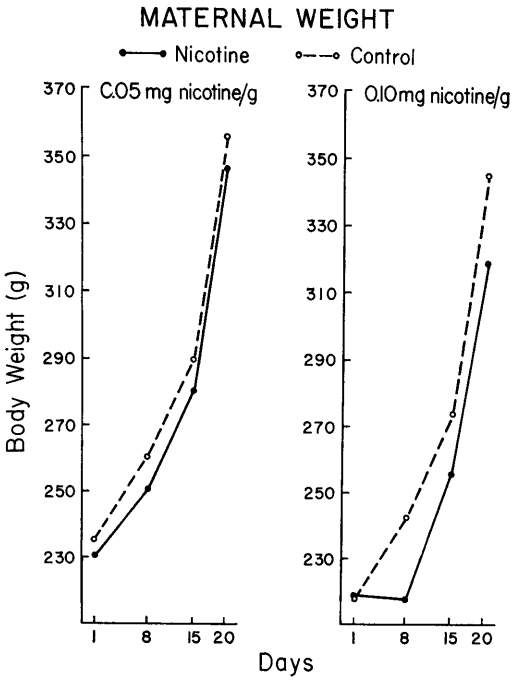

FIG. 1.

by the group on the 0.10 mg/g diet. This is reflected in the delay in maternal weight gain in this group (Fig. 1). The nicotine intake of the 0.05 mg/g group, 2.55 mg/kg/day, thus is nearly the same as that in our earlier report in which the rats received nicotine in their drinking water in a concentration of 0.05 mg/ml. The nicotine intake of the 0.10 mg/g group was much higher.

The absence of effect on fetal weight and length indicates that nicotine *per se* was not responsible for the low newborn weight in the earlier report. In view of the decrease in water intake it is likely that food intake was decreased as well. Chow and Lee showed that the maternal weight gain might remain normal in the face of food restriction in pregnant rats(3).

It has been well established that decreased birth weight is related to smoking during pregnancy in the human(3,4,5). Our observations cannot be related to these at present because of the species difference, the different method of administering nicotine, and the possibility that some other factor in tobacco smoke than nicotine may influence fetal development. One can suggest from our findings that diet should be controlled in evaluating the effect of smoking on fetal growth.

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1. Mosier, H. D., Jr., Armstrong, M. K., Proc. Soc. Exp. Biol. Med., 1964, v116, 956.
2. Chow, B. F., Lee, C., J. Nutr., 1964, v82, 10.
3. Zabriskie, J. R., Obsteet. Gynec., 1963, v21, 405.
4. Golstein, H., Goldberg, I. D., Frazier, T. M., Davis, G. E., Public Health Rep., 1964, v79, 553.
5. Underwood, P., Hester, L. L., Lafitte, T., Jr., Gregg, K. V., Am. J. Obstet. Gynec., 1965, v91, 270.

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Rubella Virus Complement-Fixing Antigen: Sedimentable and Non-sedimentable Antigenic Components. (31945)

AKINYELE FABIYI, GARY L. GITNICK, AND JOHN L. SEVER
Section on Infectious Diseases, Perinatal Research Branch, National Institute of Neurological Diseases and Blindness, and Laboratory of Infectious Diseases, National Insitute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

In studies on rubella virus and rubella virus infections several serological methods have been developed. Some of these methods include the neutralization test by the direct inhibition of CPE in tissue culture(1), the inhibition of CPE from a challenge virus by the interference technique(2-4), the fluorescent antibody test(5), and recently the complement-fixation (CF) test(6-9). Each of these tests has its inherent difficulties: notably, the time required for the neutralization test, the complexities of the FA test, and the high costs and low sensitivity of the CF test. Two CF antigen preparations have been

made: the cell-associated antigen as described by Sever *et al*(6) and Schell *et al*(8) and the concentrated fluid overlay from infected cells allowed to go to complete CPE as reported by Schmidt and Lennette, and Veronelli and Eckert(7,9). The latter preparation requires chemical precipitation and/or concentration followed by ether treatment. This antigen was soluble and was not sedimentable by high speed centrifugation. Fabiyi *et al*(10) have described certain characteristics of the antigens found in packed cell preparations.

More recently, we prepared and concentrated rubella virus specific antigens found in infected baby hamster kidney tissue cultures (BHK-21) by a relatively simple process. This paper describes the methods used. Two types of rubella CF antigens were encountered, one sedimentable and the other non-sedimentable.

Materials and methods. Virus and cell cultures. The RV strain(6) of rubella virus adapted to the continuous rabbit kidney (RK-13) cell line was used. The virus had 6 passages in African green monkey kidney tissue culture cells, and 10 passages in RK-13 cells. All tissue experiments were carried out in a continuous line of baby hamster kidney (BHK-21) which had been subcultured between 50-100 passages and which were obtained from a commercial source.* Growth and maintenance media for the BHK-21 cell line consisted of 87% Eagle's minimum essential medium in Hanks' balanced salt solution, 10% fetal calf or agamma newborn calf serum, 1% glutamine, 2% penicillin and streptomycin mixture (100 units and 100 milligrams, respectively), and phenol red. Cells were grown and maintained at 34°C.

Complement-fixation test. The microtechnique complement-fixation test was used(11). Antigen titrations were carried out in twofold dilutions, with 0.025 ml volumes. Sera used were acute and convalescent sera from proven cases of rubella virus infection. The sera were inactivated at 56°C for 30 minutes and diluted in veronal buffered saline containing calcium and magnesium ions as described by Kabat and Mayer(12).

Preparation of rubella virus complement-fixing antigen. Confluent BHK-21 tissue culture cells in 32-oz bottles or in 8-oz bottles were used. Cells in maintenance medium were infected with 2 ml (or 1 ml for 8-oz bottles) of rubella virus with a $TCID_{50}$ $\log_{10}$ of 6 or greater. Uninfected bottles served as controls. Infected cells were incubated at 34°C until harvested. For this preparation of complement-fixing antigen, infected cells were harvested after 4 days. The method of harvesting has been described(6). Briefly, this included removal of supernatant fluid, scraping of the cells with a rubber policeman, and the suspension of the cells in a small volume of supernatant fluid, all of which were pooled. and the suspended cells were centrifuged at 600 *g* for 10 minutes. A 10% suspension of the packed cells was prepared in the supernatant fluid. The cells were then frozen and thawed 3 times; the resultant preparation constituted the crude complement-fixing antigen.

Ether treatment. Two volumes of antigen were mixed with one volume of fresh anesthetic ether with the aid of a magnetic mixer at 4°C. The preparation was placed at room temperature until separation into phases occurred. Treatment took approximately 30 minutes. The details of this method have been described(13).

Results. Effect of ether treatment on rubella CF antigen. Crude 10% suspension of rubella virus CF antigen was treated with ether for various time intervals. Treatments were performed for 5, 10, 15, 20, 25, and 30 minutes. In addition, 2 serial 30-minute, 3 serial 30-minute, and 3 serial 2-hour treatments were performed. Three phases of separation were noted: a top clear ether layer, a middle emulsion layer, and an opalescent aqueous layer. Centrifugation of the ether-antigen mixture at 1500 RPM at 4°C for 15 minutes in an International Refrigerated Centrifuge as carried out by Schmidt and Lennette(7), showed 3 similar layers except that the emulsion layer occupied less space (more constricted) and the bottom aqueous phase was clearer and not opalescent. The 3 layers obtained after ether treatment were removed into separate containers, and were placed in a glass desiccator overnight at room

*Microbiological Associates, Inc., Bethesda, Md.

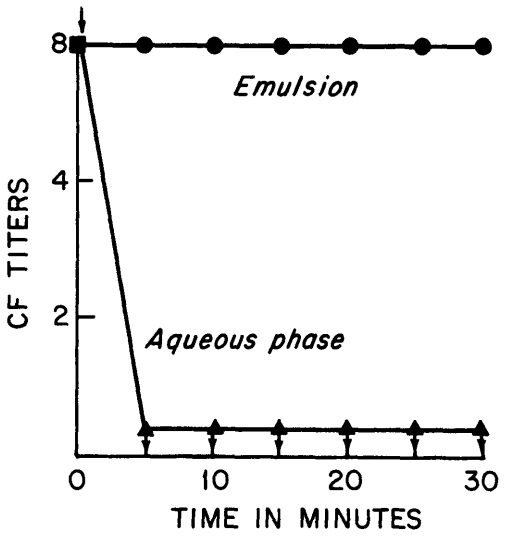

FIG. 1. Effect of ether treatment on crude rubella virus cell-associated CF antigen. Antigen was treated with ether for 5, 10, 15, 20, 25 and 30 min. CF titers are expressed as reciprocals of dilutions. Arrow shows the original titer of the crude 10% "packed-cell" suspension.

temperature to remove residual ether. There was no significant loss in CF antigen titers in preparations left overnight at either 4°C or room temperature. The emulsion layer after resuspension in veronal buffered saline to the original volume was subjected to subsequent ether treatments. The aqueous layer from the original treatment was again treated with ether. Neither the aqueous layer antigen nor the emulsion layer antigen was infectious when tested in primary African green monkey kidney tissue cultures.

The results of treatment of CF antigen with ether for various time intervals are shown in Fig. 1 and Tables I and II. Results of serial treatments for 30-minute periods and three 2-hour periods are shown in Table II. A low level of detectable CF activity was noted in the aqueous phase with a low-titered crude antigen, but when a crude antigen with a titer greater than 1:8 was used, higher CF activity was obtained in the aqueous phase. Serial treatments of the resuspended emulsion layer showed that most of the CF activity remained in the emulsion layer but some of the CF activity was released into the aqueous phase. Retreatment of the aqueous layer, however, did not reveal CF activity in the emulsion layer, and all of the CF activity originally present in the aqueous layer remained unaffected.

"Solubility" of the antigen. In an attempt to determine if rubella CF antigen was a "soluble" antigen in the sense that this term is used to describe the internal "S" components of myxoviruses, the antigen preparations (crude, emulsion layer, aqueous layer, and those preparations which were re-treated) were subjected to high-speed centrifugations for various time periods. Centrifugation was at 120,000 × *g* in a Spinco Model L Ultra-

TABLE I. Effect of Ether Extraction, Re-Extraction and High Speed Centrifugation.

	CF titer before sedimentation	CF titer after sedimentation*	
		Supernatant	Sediment
Crude antigen	8	2	4
Ether extracted antigen			
Emulsion layer	4	1 (1 hr) 1 (3 hr)	4 (1 hr) 4 (3 hr)
Aqueous layer	2	2	<1
Re-extraction of the emulsion layer			
Emulsion layer	4	<1	4
Aqueous layer	2	2	<1
Re-extraction of the aqueous phase			
Emulsion layer	<1	<1	<1
Aqueous layer	2	2	<1

* All centrifugations were carried out at 120,000 × *g*.

TABLE II. Results of Serial Ether Treatments.

Antigen preparations	CF titer*		
	Crude antigen	Emulsion layer	Aqueous layer
Crude antigen (Lot #1)	8		
First 30 min extraction†		8	2
Second 30 " " †		4	2
Third 30 " " †		4	2
Fourth 30 " " †		2	2
Crude antigen (Lot #2)	64		
First 2 hr extraction†		64	4
Second 2 " " †		8	8
Third 2 " " †		8	4

* Expressed as reciprocals of dilutions.

† Each new treatment was carried out on a previous emulsion phase antigen (in the same series) resuspended to the volume obtained after the first extraction of the crude antigen.

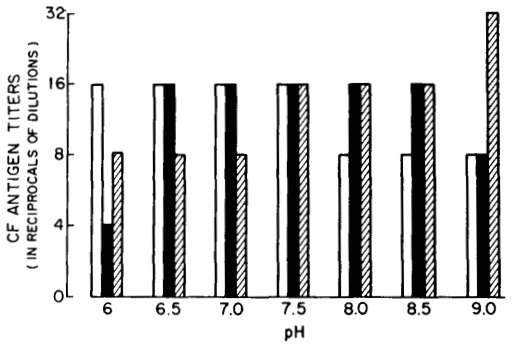

FIG. 2. Effect of pH on ether treatment of rubella CF antigen preparations. Crude antigen = Blank bars, Emulsion layer antigen = Black bars, Aqueous layer antigen = Striped bars.

centrifuge with a 39 SW head, at 4°C without brake. After centrifugation the upper two-thirds of the supernatant fluid was removed and the rest discarded. The pellet was resuspended in veronal buffered saline to the original volume.

The results of the various centrifugations on the different preparations of rubella virus CF antigen are shown in Table I. Crude antigen showed a sedimentable fraction containing most of the antigen, while a non-sedimentable fraction remained in the supernatant. The emulsion layer of ether treated rubella CF antigen contained sedimentable antigen, while the aqueous layer antigen remained in the supernatant, confirming that the aqueous layer antigen was non-sedimentable when subjected to high speed centrifugation for 1 to 3 hours.

Effect of pH on ether treatment of CF antigen. The effect of pH on the ether treatment of crude CF antigen before and during the procedure was next investigated. This study was designed to determine if alterations in pH would enhance the CF titers obtained after ether treatment of such preparations. Adjustments of pH were accomplished with 10% acetic acid, 10% sodium bicarbonate up to pH 8, and 1 N-sodium hydroxide for pH's beyond 8. Crude CF antigen with an initial pH of 7.0 was adjusted to give aliquots with pH's of 6, 6.5, 7.5, 8, 8.5 and 9. Ether treatment of each was carried out immediately as described after pH adjustments and the emulsion and aqueous layers were dried and resuspended to original volumes in veronal buffered saline. Control crude antigen was similarly treated.

The results of ether treatment of rubella CF antigen at various pH's are shown in Fig. 2. There was no effect of pH changes on CF titers of the crude antigen from pH 6 through pH 7.5, however, alkaline pH's (8 to 9) lowered the titer of the crude antigen. Treatment of crude CF antigen at pH 9 enhanced the CF titer by fourfold in the aqueous layer. At this pH the CF titer of the crude antigen and of the emulsion layer antigen were depressed. In general these results showed that emulsion layer antigen could be obtained without loss in titer at pH's 6.5, 7.0, 7.5, 8.0 and 8.5.

Specificity of rubella CF antigen preparations. Specificity of the rubella CF antigen preparations was determined using human convalescent sera from confirmed cases of Influenza A, Parainfluenza 2 and 3, mumps, respiratory syncytial virus, and mycoplasma hominis (PPLO). The results of the CF test with these sera using rubella CF crude, emulsion-layer and aqueous-layer antigens are shown in Table III. The rubella CF antigens did not react with the convalescent respiratory virus antisera employed, but did fix complement in the presence of convalescent rubella serum.

Ether treatment of RNA and DNA viruses. Rubella virus has been described as a myxovirus(14,15). For comparisons the CF antigens of 2 myxoviruses (RNA viruses) and of a DNA virus were treated with ether in the same manner as described above. Viruses used were Influenza A/305/Japan (1957), mumps and herpes simplex. The influenza, mumps, and herpes simplex viruses were grown in chick-embryo. CF tests were carried out with convalescent human sera. The results of CF tests on these ether-treated antigens are shown in Table IV. No detectable CF activity was obtained in the emulsion layer of the ether treated influenza antigen while considerable CF activities were noted in the emulsion layers of mumps and herpes simplex ether treated CF antigens.

The experiments discussed herein were each repeated at least 4 times with very similar results.

TABLE III. Reaction of Rubella CF Antigen Preparations with Antiserum to Respiratory Agents.

Respiratory antisera	Homologous antigen	CF titers* Rubella antigens Crude antigen	Emulsion layer	Aqueous layer
Influenza A	16	0	0	0
Parainfluenza 2	16	0	0	0
Parainfluenza 3	16	0	0	0
Mumps	8	0	0	0
Respiratory syncytial	16	0	0	0
Mycoplasma hominis	16	0	0	0
Rubella	8	8	4	2

* Expressed as reciprocals of dilutions. 0 = Titer <1:4.

Discussion. The infective moieties of rubella virus as well as those of other myxoviruses have been shown to be sensitive to ether treatment(2-4,16-18). Ether treatment of purified influenza virus, for example, results in the inactivation of infectivity, and the release of viral internal soluble ("S") CF antigen, strain specific CF or V antigen as well as viral hemagglutinins(16-18).

In the present paper, the original material is not purified virus but rather virus-infected cells which fix complement in the presence of rubella virus specific antiserum. Ether treatment of this antigen has, however, resulted in two preparations both of which can fix complement in the presence of specific rubella antiserum, but only one of which is not sedimentable and may be compared to the "S" antigen of the myxoviruses. It is significant that soluble non-sedimentable rubella antigen has also been prepared from concentrated ether treated rubella virus in infected tissue culture fluids(7,9). This soluble rubella CF antigen is present in the aqueous layers of both ether treated "cell pack" CF antigen (as reported here) and concentrated infected virus fluid(7,9). However, only the ether treated rubella "cell pack" CF antigen has in addition a sedimentable complement-fixing preparation which is present in the emulsion layer of ether treated "cell pack" antigen.

TABLE IV. Ether Extraction of RNA and DNA Viruses.

Viral antigen	CF titer* Crude antigen	Emulsion layer	Aqueous layer
Influenza A	8/512	0/0	2/64
Mumps	8/128	4/32	4/32
Herpes simplex	32/32 †	16/64	2/4

* CF titers expressed as reciprocals of dilutions.
† Numerator = Antigen titer.
Denominator = Serum titer.
0 = Antigen titer <1:1 (undiluted).

The data presented here also show that prolonged and repeated ether treatments of the sedimentable emulsion layer antigen result in the release of more soluble CF antigen into the aqueous layer. Furthermore, ether treatment of crude cell pack antigen at pH 9.0 releases more soluble CF antigen into the aqueous phase. This suggests that the sedimentable antigen preparation is virus antigenic matter associated with or attached to cellular component material, since the crude CF antigen originates as a "cell pack" preparation. Whether or not this antigen comes from whole virus within the infected cell or from pools of viral protein unattached to virus nucleic acid is yet to be determined.

Summary. Ether treatment of rubella virus "cell pack" antigen resulted in the production of specific sedimentable and non-sedimentable ("soluble") complement-fixing preparations which were serologically indistinguishable when tested with convalescent human sera. The sedimentable antigen was found in the emulsion phase, while the "soluble" antigen was found in the aqueous phase. None of these antigens was found to be infectious in tissue culture. Prolonged and repeated ether treatments of the sedimentable antigen resulted in the release of more "soluble" antigen into the aqueous phase. Maximum "soluble" antigen titers were produced when crude cell pack antigen was ether treated at pH of 9. The rubella virus speci-

ficity of the sedimentable and non-sedimentable CF antigen preparations were established. The non-sedimentable CF preparation was compared to myxovirus soluble antigen in the sense that this term was used to describe the internal CF antigen of the latter. The sedimentable rubella CF antigen was considered to be "soluble" antigen attached to or associated with cellular material.

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1. Plotkin, S. A., Dudgeon, J. A., Ramsay, A. M., Brit, Med. J., 1963, v2, 1296.
2. Weller, T. H., Neva, F. A., Proc. Soc. Exp. Biol. Med., 1962, v111, 215.
3. Parkman, P. D., Buescher, E. L., Artenstein, M. S., ibid., 1962, v111, 225.
4. Sever, J. L., Schiff, G. M., Traub, R. G., J.A.M. A., 1962, v182, 663.
5. Brown, G. C., Maasab, H. F., Science, 1964, v145, 943.
6. Sever, J. L., Huebner, R. J., Castellano, G. A., Sarma, P. S., Fabiyi, A., Schiff, G. M., Cusumano, C. L., ibid., 1965, v148, 385.
7. Schmidt, N. J., Lennette, E. H., Proc. Soc. Exp. Biol. Med., 1966, v121, 2.
8. Schell, K., Huebner, R. J., Turner, H. C., ibid., 1966, v121, 41.
9. Veronelli, J. A., Eckert, E. A., ibid., 1966, v121, 1223.
10. Fabiyi, A., Sever, J. L., Ratner, N., Kaplan, B., ibid., 1966, v122, 392.
11. Sever, J. L., Huebner, R. J., Castellano, G. A., Bell, J. A., Am. Rev. Resp. Dis., 1962, v85, 342.
12. Kabat, E. A., Mayer, M. M., Experimental Immunochemistry, C. C Thomas, Springfield, Ill., Chap. 41, 1962.
13. Lief, F. S., Henle, W., Virology, 1956, v2, 753.
14. Norrby, E., Magnusson, P., Friding, B., Gard, S., Arch. fur die Gesamte Virusforsch., 1963, v13, 4.
15. Phillips, C. A., Behbehani, A. M., Johnson, L. W., Melnick, J. L., J.A.M.A., 1965, v191, 615.
16. Hoyle, L., J. Hyg., 1952, v50, 229.
17. Fabiyi, A., Lief, F. S., Henle, W., J. Immunol., 1958, v81, 467.
18. Davenport, F. M., Hennessy, A. V., Drescher, J.,Webster, R. G., Ciba Foundation Symposium on Cellular Biology of Myxovirus Infections, Little, Brown & Co., Boston, Mass., 1964, p272.

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Mitosis-Stimulating Factor in Partially Hepatectomized Rats as Affected by Adrenalectomy and Dexamethasone.* (31946)

S. S. CARDOSO, A. L. FERREIRA, A. C. M. CAMARGO, AND H. CALDO (Introduced by A. D. Welch)
Departments of Pharmacology and Morphology, Faculty of Medicine of Ribeirão Prêto, Univ. of São Paulo, Brazil

The existence in plasma of partially hepatectomized rats of a factor that stimulates mitosis in the liver of normal rats was first demonstrated by 3 groups: Christensen and Jacobsen(1), Bucher *et al*(2), and Wenneker and Sussman(3). Subsequent work confirmed these findings(4-6) and also demonstrated that after partial hepatectomy mitosis is stimulated in other tissues as well as in the liver (6); this finding suggested a possible relationship between this mitosis-stimulating factor and the mechanism of control of normal cell division and organ size(7). These studies led to attempts to demonstrate and identify in the plasma of hepatectomized rats such a mitosis-stimulating factor or factors. The results reported in the literature are conflicting, with findings indicating that the administration of serum obtained from partially hepatectomized rats (a) stimulates mitosis in the liver of normal rats(4,5) or (b) has no effect at all(8).

The absence of important endocrine glands such as the pituitary does not prevent the appearnce of the mitosis-stimulating factor in cortisone-treated, partially hepatectomized

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