stable at −70°C and +4°C. The hemagglutinin was used to detect rises in HI antibody in paired serum specimens. Hemagglutination inhibition antibodies were present in titers of 1:40 to 1:320, 2 to 3 years after rubella infection.

The supervision of all tissue culture preparation by Mrs. Anna Hall and the excellent technical assistance of Miss Marianne Davis and Miss Ludie Holland are gratefully acknowledged.

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Rubella Complement Fixing Antigen Prepared by Alkaline Extraction Of Virus Grown in Suspension Culture of BHK-21 Cells. (32039)

P. E. HALONEN,* H. L. CASEY, J. A. STEWART, AND A. D. HALL
(Introduced by G. R. Cooper)
Virus Reference Unit, Virology Section, National Communicable Disease Center, Bureau of Disease Prevention and Environmental Control, Public Health Service, U. S. Department of HEW, Atlanta, Ga.

Schmidt and Lennette(9) have adapted an alkaline extraction method for preparation of rubella complement fixing antigen using monolayer cultures of BHK-21 cells. A technique for the growth of high titered rubella virus in suspension culture of the same cells has recently been reported by Vaheri *et al* (13).

The present report describes the preparation of rubella complement fixing (CF) antigen with alkaline extraction of virus grown in suspension culture of BHK-21 cells. Also reported are the high levels of antibody and their duration, measurable with this type of rubella antigen.

Materials. Virus strains. The rubella virus strain RA 27/3(13) for CF antigen preparation was kindly supplied by Dr. Antti Vaheri, Wistar Institute of Anatomy and Biology, Philadelphia, Pa. When received, it had been passaged 4 times in WI-38 cells and about 25-30 times in BHK-21/13S cells. In this laboratory 8 more passages were made in the latter cells.

The Gilchrist strain of rubella virus used for neutralization tests was obtained from Dr. J. L. Sever, National Institutes of Health, Bethesda, Md. Its previous history is unknown, but in this laboratory it was passaged in primary or continuous African green monkey kidney (GMK) cells. It was used in neutralization tests at the 10th and 11th passage level.

Cell cultures. Clone 13S of BHK-21 cells for suspension culture was supplied by Dr. Antti Vaheri and was maintained according to the technique reported(13) with minor modifications. The growth medium, called here Wistar growth, was Eagle's minimum essential medium prepared in Hanks' solution with a double concentration of amino acids (except 1 X glutamine) and vitamins, supplemented with 0.1 mg per liter of ferric nitrate, 4.5 g per liter of glucose (final concentration), 10% tryptose phosphate broth

* On leave of absence from Dept. of Virology, Univ. of Turku, Turku, Finland.

(Difco)† and 10% heat inactivated fetal calf serum (Hyland Laboratories, Los Angeles, Calif.). The maintenance medium, called here Wistar maintenance, was the same but with 2% fetal calf serum.

Monolayer cultures of BHK-21/13S cells were grown in 32 oz prescription bottles with 60 ml of Wistar growth medium and trypsinized twice a week. From each bottle of tissue culture 6 bottles were prepared. For the suspension culture, bottles of cells were grown for 3 to 4 days. Cells from 8 to 9 bottles were trypsinized, and an 800 ml suspension culture containing about 4×10^8 cells was prepared in Wistar growth medium in a one liter Erlenmeyer flask with cotton plugged side arm and screw cap. A small magnetic rod with central ring was placed in the flask, and the suspension was incubated at 37°-38°C (temperature measured inside of the flask) with constant spinning on a magnetic stirrer.

For neutralization tests, monolayer tube cultures of primary GMK were prepared by Biological Reagents Section, NCDC. Cells were grown for 4 days in a medium consisting of 0.5% lactalbumin hydrolysate in Hanks' solution and supplemented with 5% fetal calf serum. The maintenance medium was a modified Eagle's medium(5) with 2% fetal calf serum.

Each lot of fetal calf serum for either antigen preparation or neutralization test was pretested for heat stable rubella virus inhibitors. Some lots of fetal calf serum were found to decrease virus titers by 1.5 to 2.0 log units.

Methods. Complement fixing antigen. A one-day-old suspension culture of BHK-21/13S cells was transferred to a 2 liter Erlenmeyer flask with side arm and screw cap and inoculated with 100 ml of undiluted virus having a titer of about 10^7 $TCID_{50}$ per ml. Fresh virus from a previous harvest was usually used without freezing and thawing. After 2 hours incubation at 35°C without spinning but with occasional shaking, an equal volume (800 ml) of Wistar maintenance was added. The rubella infected suspension culture of about 1700 ml volume (with final fetal calf serum concentration of 6%) was incubated for 5 to 7 days at 35°C with constant spinning. The pH of the culture was checked daily with indicator paper and was adjusted to 7.0 to 7.2 with 5% $NaHCO_3$. During the first 3 to 4 days when CO_2 production was copious, the screw cap was left partly open to decrease the volume of $NaHCO_3$ required to adjust the pH.

To harvest, the culture was centrifuged for 30 minutes at 2000 RPM (International Centrifuge, Model PR-2, Head No. 259). The cell sediment was resuspended in sufficient 0.1 M glycine-NaOH buffer, pH 9.0(4) to make a 10% suspension. This suspension was incubated for 8 hours at 35°C with occasional shaking, sonicated in a Raytheon sonic oscillator (Model No. DF 101) at 10 kc for 5 minutes, and then centrifuged in a Spinco for 30 minutes at 10,000 RPM (Rotor No. 30). The supernatant fluid is the CF antigen which was stored at +4°C.

Complement fixation test. The micro LBCF technique(2) was used. Checkerboard titration of each antigen were performed using known positive and negative human sera. A hyperimmune rhesus monkey serum (kindly supplied by Dr. F. A. Murphy, Developmental Virology Unit, Virology Section, NCDC) was also used for many of the antigen titrations.

Neutralization test. The indirect neutralization test of Parkman *et al*(7) with ECHO-11 as challenge virus was used with minor modifications. The diluent for virus and serum dilutions was Hanks' solution with 1% bovine albumin. The virus and serum mixtures were incubated for 1 hour at room temperature before inoculation into GMK tissue culture tubes. Three culture tubes for each 2-fold serum dilution were inoculated. In the control titration of virus, 6 tubes per each half log dilution of virus were used. After 2 days incubation at 36°C, 1.0 ml of fresh maintenance was added to all tubes, and they were incubated for 3 more days. On the 5th day, the medium was poured off and the GMK tubes were challenged with 1000 $TCID_{50}$ of ECHO-

† Use of trade names is for identification only and does not constitute endorsement by the Public Health Service, U. S. Dept. of HEW.

11 virus in 1.0 ml of maintenance. After the tubes were incubated for 3 more days, the tests were read.

Two dilutions of virus were used in each test with each serum specimen. Serum titers were those obtained using that dilution of virus which contained about 30 to 50 ID_{50}.

Results. Sensitivity and specificity of the antigen. Representative CF antigen titrations of one lot of alkaline extracted antigen tested against a late convalescent human serum and a hyperimmune monkey serum are shown in Table I. The antibody titers were considerably higher with alkaline extracted CF antigen than with the cell pack antigen(10) from Microbiological Associates, Inc. (MBA). These titrations indicate that the undiluted alkaline extracted antigen may not be sufficiently potent to represent a true optimal dilution of antigen. An optimal dilution is normally defined as that dilution which gives the highest CF antibody titer with a specific antiserum(2). Thus, if the alkaline extracted antigen were more concentrated, it might be possible to see the optimal dilution of antigen at 1:2 or 1:4 and detect even higher antibody titers in convalescent serum. Some later CF antigen lots have given the same serum titers with antigen diluted 1:4, but these lots were anticomplementary (AC) in lower dilutions; therefore, the optimal dilution of antigen could not be determined. Alkaline extracted antigens did not react with the negative control serum, nor did the control antigen prepared from uninoculated suspension cultures of BHK-21/13S cells by alkaline extraction react with rubella positive serum.

TABLE I. A Representative Titration of Alkaline Extracted Rubella Complement Fixing Antigen Tested Against Human and Monkey Serum.

Antigen	Late convalescent human immune serum						Hyperimmune monkey serum					Complement controls CH_{50}		
	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:16	1:32	1:64	1:128	1:256	1:512	5	2.5	1.25
Alkaline extracted																
Undiluted	4	4	4	4	4	+|-	0	4	4	4	4	3	0	0	+|-	3
1:2	4	4	4	4	+|-	0	0	4	4	4	4	2	0	0	0	2
1:4	4	4	4	+|-	0	0	0	4	4	4	3	0	0	0	0	2
1:8	+|-	0	0	0	0	0	0	3	3	+|-	0	0	0	0	0	2
1:16	0	0	0	0	0	0	0	+|	0	0	0	0	0	0	0	0
MBA*																
1:2	4	4	2	+|	0	0	0	4	4	4	3	0	0	0	0	2
MBA control																
1:2	0							0						0	+|	4
Serum control	0							0								

* Rubella (German measles) CF antigen, Lot 3-220, titer 1:8, Microbiological Associates, Inc., Bethesda, Md.

The two types of CF antigens are further compared in Table II. Acute and convalescent sera were collected from young adults during a rubella epidemic. CF antibody titers obtained with alkaline extracted antigen are in most cases considerably higher than those with the MBA cell pack antigen and correlate well with the appearance of neutralizing antibody.

When infected suspension cultures were removed from the flask after 5-7 days growth of virus and treated only by freezing and thawing 3 times and used as antigen, they showed the same antibody titers as did the cell pack antigen of MBA.

The supernatant fluid (fluid phase), after harvest of infected cells by centrifugation, was concentrated 100-fold by dialysis against polyethylene glycol(8). This antigen also reacted specifically with convalescent rubella serum; however, antibody titers were considerably lower than with the alkaline ex-

TABLE II. Complement Fixing Antibody Titers Obtained with Alkaline Extracted Rubella Antigen and Cell Pack Antigen of Microbiological Associates, Inc. (MBA) and Neutralization Titers of Serum Specimens Collected from Young Adults During a Rubella Epidemic.

Specimen No.		Days after onset of rash	CF antibody titer: Alkaline extracted antigen	CF antibody titer: MBA	Neutralization titer
298	S1	1	<4	8	<8
	S2	8	≥256	24	8
	S3	22	≥256	48	16
299	S1	3	<4	<8	<8
	S2	10	64	16	16
	S3	24	64	8	8
300	S1	2	<4	<8	<8
	S2	9	256	12	16
	S3	23	256	16	32
301	S1	0	<8	<8	NT
	S2	7	128	16	32
	S3	21	128	32	32
302	S1	2	<4	<8	<8
	S2	9	64	8	8
	S3	23	256	32	16
303	S1	2	<4	<8	<8
	S2	9	8	6	8
	S3	23	128	32	NT
304	S1	1	<8	<8	<8
	S2	8	4	8	NT
	S3	22	64	16	32
394	S1	1	<4	<8	<8
	S2	22	64	≥64	8
395	S1	2	<4	<8	<8
	S2	20	32	16	128
396	S1	3	<4	8	<8
	S2	21	64	32	8
397	S1	0	<4	<8	<8
	S2	21	32	24	16
398	S1	2	<4	<8	<8
	S2	23	64	12	64
399	S1	4	<4	<8	8
	S2	22	64	16	32

tracted antigens but similar to those obtained with MBA antigen.

The alkaline extracted antigens gave reproducible CF antibody titers from day to day. A positive human serum was used with a single lot of alkaline extracted antigen as a control for rubella CF tests on 6 occasions. The antibody titer was 1:16 on 1 occasion, 1:32 on 3 occasions and 1:64 on 2 occasions. The same lot of antigen tested with immune monkey serum gave antibody titers of 1:128 on 4 occasions, 1:256 on 2 occasions and 1:512 on 1 occasion.

Persistence of antibodies. For up to 2 years after rubella infection in children, CF antibody as determined with alkaline extracted antigen decreased slowly as shown in Table III. This type of CF antibody apparently persists several years since titers of 1:4 to 1:32 were found as long as 2 years after illness. The persistence of CF antibody was

TABLE III. Rubella CF Antibody Titers of Sera Collected from a Children's Home* at Intervals Following a Rubella Epidemic 2 Years Previously. An alkaline-extracted antigen was used.

Child No.	Time of rubella illness	Time of specimen collection	CF antibody titer
908	April, 1964	Jan., 1964	<4
		Sept., 1964	16
		Jan., 1965	16
		Oct., 1965	16
988	April, 1964	Mar., 1964	<4
		Sept., 1964	16
		Oct., 1964	32
		Oct., 1965	4
		Mar., 1966	4
		Aug., 1966	4
1041	May, 1964	Sept., 1964	64
		Feb., 1965	32
		Oct., 1965	32
		Mar., 1966	32
		Aug., 1966	16
1042	May, 1964	May, 1964	8
		June, 1964	32
		Aug., 1964	32
		Oct., 1965	32
		Aug., 1966	16
2061	May, 1964	Mar., 1964	<4
		Aug., 1964	64
		Dec., 1964	32
		Oct., 1965	16
		Apr., 1966	16
		Aug., 1966	16
2062	May, 1964	Mar., 1964	<4
		Aug., 1964	64
		Dec., 1964	32
		Oct., 1965	16
		Apr., 1966	16
		Aug., 1966	16
2072	June, 1964	May, 1964	<4
		June, 1964	4
		Dec., 1964	8
		Oct., 1965	4
		Aug., 1966	4
2081	May, 1964	Aug., 1964	128
		Sept., 1964	64
		Dec., 1964	32
		Feb., 1965	64
		Oct., 1965	32

* Sera obtained in a respiratory illness study conducted by the Respirovirus Unit, Virology Section, NCDC.

also demonstrated in a survey of serum specimens collected from females in the Atlanta area. In groups tested between the ages of 6 and 29, the CF test was positive in 75% to 90% of the individuals tested. In the age group 30-35 approximately 65% of the women were positive. While it may be that antibody persists from childhood, it is possible that antibody titers are boosted through reinfection(1).

Stability of the antigen and anticomplementary activity. To test the stability of the alkaline extracted antigen, a single lot of CF antigen was stored at 35°C for 7 days, at 22°C for 3 weeks, and at 4°C for 12 weeks, and at —70°C for 12 weeks. No decrease in the antigen titer was noted at any of these temperatures within the indicated time periods. This same lot of antigen was frozen and thawed 10 times without decrease in its reactivity.

The first few lots of antigen had no detectable AC activity. However, antigens prepared a few months later showed AC activity at low dilutions. When earlier passage virus inoculum and earlier passage BHK-21/13S cells were used the AC activity disappeared completely. The AC activity may have been caused by some mycoplasma contamination even though several attempts to grow these microorganisms have failed. Support for possible mycoplasma contamination was provided by an electron microscopy study of inoculated cells of suspension culture which showed mycoplasma-like particles within the cells(6).

Discussion. Since the original demonstration of specific rubella virus CF antigen in a cell pack from infected RK-13 cells by Sever *et al*(10), preparation of rubella CF antigens in various cell culture systems by other techniques has been reported(3,8,9,12,14). The CF antibody titers of convalescent serum specimens appear to be rather low when tested with cell pack antigens(10,11,12) or concentrated fluid phase antigens(8,14). Low titers were also recorded in the present study with a commercial cell pack antigen whereas the alkaline extracted CF antigens prepared from infected BHK-21/13S cells grown in suspension showed considerably higher antibody titers. Therefore, alkaline extracted antigens are more practical for serological diagnosis of rubella. The additional advantage of the alkaline extracted CF antigen is its great stability at —70°C, —20°C, +4°C and even at room temperature.

Wong and Belcourt(15) were able to show rubella complement fixing antigen in unconcentrated infected tissue culture fluids while others(7,8,10) have been unable to demonstrate it. In the present study the unconcentrated infected suspension culture fluids had the same CF reactivity as commercial cell pack antigen. Even the fluid phase which is discarded during preparation of the alkaline extracted antigen has specific activity when concentrated. This may indicate that even more rational techniques for preparing rubella CF antigen should be explored.

Alkaline extraction followed by sonication apparently releases an antigen or antigens from the cells or from virus particles which are possibly not present in other preparations and which may be necessary for obtaining high serum titers with convalescent phase specimens. Gradient centrifugation experiments may reveal more information concerning the nature of alkaline extracted rubella CF antigen.

Summary. Alkaline extracted rubella complement fixing antigen was prepared from infected BHK-21/13S cells grown in suspension culture. Although antigen potent enough to ensure optimal dilutions may not have been used, the complement fixing antibody titers of convalescent phase specimens were considerably higher than they were when commercial cell pack antigen was used. They were also higher than the titers reported earlier with the use of other types of rubella complement fixing antigens. The alkaline extracted antigens were stable at —70°C, —20°C, and +4°C.

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Uncoupling of Oxidative Phosphorylation by Ultrafiltrates Of Uremic Serum. (32040)

ROBERT P. GLAZE, JEAN M. MORGAN, AND ROBERT E. MORGAN
(Introduced by J. M. McKibbin)
Departments of Biochemistry, and of Medicine, University of Alabama Medical Center, Veterans Administration Hospital, and Department of Biochemistry, School of Dentistry, University of Alabama Medical Center, Birmingham

Previous investigation has suggested that one or more substances toxic to tissues are present in the serum of individuals suffering from uremia(1-5). Recently, Bittar (personal communication) has shown that an ultrafiltrate of serum from uremic patients will inhibit the sodium, potassium adenosinetriphosphatase (ATPase) of crab muscle. Renner and Heintz(5) reported aberrations of metabolism in kidney cortex in the presence of factors from serum of uremic individuals, and the respiration of isolated mitochondria was thought to be uncoupled in the presence of certain protein free fractions isolated from such sera. This report presents data indicating that an ultrafiltrate of sera from uremic persons does indeed produce a partial uncoupling, and further that this uncoupling appears to be associated only with adenosinetriphosphate (ATP) production associated with electron transport between reduced nicotinamide-adenine dinucleotide (NADH) and cytochrome *b* (Site I).

Materials and methods. All studies were carried out on rat liver mitochondria isolated by the Schneider and Hogeboom technique from a 10% (w/v) 0.25 M sucrose homogenate of rat liver(6). The twice washed mitochondria were suspended at a concentration of 50 mg protein/ml in 0.25 M sucrose before use. The respiration of the isolated mitochondria was studied using a Clark oxygen electrode connected to appropriate amplifiers and recorders(7).

Normal and uremic serum ultrafiltrates were obtained by centrifugation through a cellophane membrane at 4°C or by use of a Schleicher and Schuell vacuum ultrafiltration apparatus which utilizes a collodion membrane.

All patients whose sera were used had symptoms of renal failure and had blood urea nitrogen (BUN) levels of 100-300 mg % (average of 177 mg %) and serum creatinine levels of 5.4-30 mg % (average of 15 mg %). Of the 7 patients with chronic renal failure, 3 patients had diagnoses of nephrosclerosis, 3 patients had chronic pyelonephritis, and one had glomerulonephritis. Two patients had acute renal failure secondary to pneumonia and surgery. Several patients were receiving medication including