14 days than the 4 (23-26) who received 1 ml. This difference was less apparent at 28 days.

All 6 men who received adenovirus 2 group-specific antigen developed titers of neutralizing antibody, ranging from 1:2 to 1:512 at 14 days, and from 1:2 to 1:1024 at 28 days (Table III). Three men possessed substantial antibody titers at 67 or 110 days.

Discussion. Neutralizing antibody induced in adults by immunization with adenovirus soluble antigens was observed to persist for at least 4 to 12 months. Less adenovirus 1 group-specific antigen than type-specific antigen (protein) was needed to achieve comparable antibody titers in most instances. This indicates greater antigenicity of the group-specific antigen in man as measured by induction of neutralizing antibody. This result is consistent with the finding that the group-specific antigen of type 5 adenovirus produced a higher neutralizing antibody response in rabbits than did the type-specific antigen(9).

It has been postulated that the type-specific and group-specific antigens of adenovirus type 5 possess structurally different type-specific determinants and that neutralizing antibody developed in response to infection would consist of two species of antibody(9). Although the immunogenic significance of these antibodies has not been fully defined, available evidence indicates that neutralizing antibody produced in man in response to vaccination with the type-specific antigen and possibly the group-specific antigen of adenovirus 1 is protective(1). Nevertheless, additional experiments are required to ascertain the quality and duration of immunity induced by each of these antigens.

Summary. Eleven of 15 men given the type-specific antigen of adenovirus 1 and 10 of 10 men given type-specific antigen of adenovirus 2 developed significant levels of homotypic neutralizing antibody. All 17 men who were given the group-specific antigen of either adenovirus 1 or 2 developed antibody. Measurable levels of these antibodies persisted for extended periods of time.

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The Complement-Fixing Antigen of Rubella Virus.* (30748)

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Immunological studies on the rubella virus have been limited by the complexities and variables inherent in neutralization test procedures for this virus and also by the difficulties which have been encountered in demonstrating *in vitro* serologic reactions with the virus. Although the virus produces a cytopathic effect in the RK-13 line of rabbit kidney cells(1) the effect is variable from one passage to another and is usually seen only with low dilutions of the virus. Hence,

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neutralization tests are usually performed by the interference technic employing grivet monkey kidney cell cultures(2-4). Initial attempts to demonstrate viral antigen by fluorescent antibody (FA) staining or by complement fixation met with failure(4-6), but there have been recent reports of the use of the indirect FA technic for detection of antibodies in patients' sera(7,8), and Sever *et al*(9) have described a complement-fixing antigen prepared from the cellular phase of infected cell cultures. In this laboratory a complement-fixing antigen has been prepared by concentration of the fluid phase of infected RK-13 cell cultures, and this report describes the preparation and certain properties of the antigen as well as its reactivity with immune animal and human sera.

Materials and methods. Rubella virus strain. Complement-fixing antigens were prepared from the RV strain of rubella virus, obtained from Drs. J. L. Sever and G. M. Schiff, National Institute of Neurological Diseases and Blindness, Bethesda, Md. The virus was received at the 10th passage level in grivet monkey kidney cells, and was passaged in this laboratory in RK-13 cells; it was at the 12th or 13th passage level in this cell line when utilized for the preparation of antigens.

RK-13 cell line. This continuous line of rabbit kidney cells(10) was kindly supplied by Drs. Sever and Schiff, and was carried in this laboratory on a medium consisting of 10% fetal bovine serum and 90% Eagle's Minimum Essential Medium (prepared in Hanks' balanced salt solution). For viral propagation, the above medium was used with the concentration of fetal bovine serum reduced to 2%.

Cell cultures were prepared in prescription bottles of various sizes. For subpassage the cells were trypsinized and 4 new bottle cultures were prepared from each trypsinized culture (a 4:1 split). The cells were passed at weekly intervals and fed an additional one-half volume of growth medium 4 to 5 days after initiation of the cultures.

Preparation of rubella virus for use as complement-fixing antigen. Cultures of RK-13 cells were prepared in 4 oz or 16 oz prescription bottles, and when confluent monolayers were obtained the outgrowth medium was removed and replaced with maintenance medium (8 ml for 4 oz bottles, 20 ml for 16 oz bottles). Cultures in 4 oz bottles were inoculated with 0.2 ml of undiluted seed virus, and those in 16 oz bottles with 0.5 ml of virus, and incubated at 36°C for 10 days. Infected cells and fluids were harvested and subjected to 3 cycles of freezing and thawing, after which the fluids were clarified by centrifugation at 2000 rpm for 20 minutes. The clarified fluids were concentrated 100-fold by dialysis against polyethylene glycol (Carbowax 20 M†), and the concentrates were treated with Genetron or ether for use as antigens.

Genetron treatment consisted of homogenizing equal volumes of the concentrate with the fluorocarbon, Genetron 113‡ in a Virtis homogenizer at 23,000 rpm for 3 minutes. The homogenate was then centrifuged at 1500 rpm for 15 minutes and the aqueous phase was collected for use as antigen. Ether treatment consisted of adding ½ volume of anesthetic ether to the concentrates, mixing with a magnetic stirring bar for 1 hour at room temperature, and then centrifuging at 1500 rpm for 15 minutes and removing the aqueous phase for use as antigen.

Control antigens were prepared in the same manner from uninfected cultures.

Immune sera to the rubella virus. Specific immune sera to the rubella virus were prepared by intranasal inoculation of a rhesus monkey and by intravenous inoculation of rabbits.

The monkey was inoculated with 0.5 ml of undiluted virus into each nostril; a rash appeared on the 27th day after inoculation, and the animal was bled 3 weeks after the appearance of the rash.

For the preparation of immune serum in rabbits, the immunizing antigen was propagated in an RK-13 cell line grown and maintained on medium containing rabbit serum rather than bovine serum. This was done in order to avoid the production of anti-bovine antibodies which might mask the activity of

† Carbide and Carbon Chemicals Co., Union Carbide & Carbon Corp., New York.

‡ Allied Chemicals and Dye Corp., New York.

TABLE I. Demonstration of Specific Rubella Virus Complement-Fixing Antigen in Concentrates of Infected RK-13 Cell Cultures.

Antigen	CF titer* of antigen *vs* Rabbit serum: Pre-immunization	Rabbit serum: Post-immunization	Human serum: Acute phase	Human serum: Conv. phase
Infected RK-13 cell culture, unconcentrated	none†	none	—‡	—
Cells in 1/50th orig volume of fluid	"	1:1	—	—
Infected RK-13 cell culture, unconcentrated	"	none	—	—
Cells in 1/50th orig volume of fluid	"	1:1	—	—
Fluid phase, conc 50×	"	1:4	—	—
Infected RK-13 cell culture, unconcentrated	"	none	none	none
Cells in 1/100th orig volume of fluid	"	1:1	"	1:1
Fluid phase, conc 100×	"	1:8	"	1:8
Uninfected RK-13 cell culture				
Cells in 1/100th orig volume of fluid	"	none	"	none
Fluid phase, conc 100×	"	"	"	"

* Titer is based on a block titration, and is the highest dilution of antigen which gave 3-plus or 4-plus fixation with the highest dilution of immune serum.
† No specific fixation with undiluted antigen.
‡ Not done.

viral antibodies in complement fixation tests. Rabbits were inoculated by the intravenous route with 1 ml of infected cells and fluids at weekly intervals for a total of 5 injections and were bled 2 weeks after the last immunizing injection.

Complement fixation tests. Complement fixation tests were performed by our standard procedure adapted to use in the microtiter system(11).

Neutralizing antibody assays. Sera were examined for the presence of neutralizing antibodies to the rubella virus by interference neutralization tests conducted in tube cultures of the BS-C-1 line of grivet monkey kidney cells(12) or primary grivet monkey kidney cells. Sera were inactivated at 56°C for 30 minutes and 2-fold dilutions in Eagle's medium were mixed with an equal volume of rubella virus diluted to contain approximately 10 TCD_{50} per 0.1 ml. The serum-virus mixtures were incubated for 1½ to 2 hours at 4°C, and 0.2 ml volumes of each mixture were inoculated into tube cultures. The cultures were incubated at 36°C for 5 days, at which time they were inoculated with 0.1 ml of a dilution of echovirus type 11 containing approximately 100 TCD_{50} per 0.1 ml; they were read 2 and 3 days after inoculation of the echovirus. Neutralization of the rubella virus was evidenced by the cytopathic effect produced by the echovirus, while failure of the serum to neutralize the rubella virus was indicated by lack of a cytopathic effect of the echovirus (interference).

Results. Demonstration of rubella CF antigen in concentrates of infected cell culture fluids. Because other investigators had reported inability to detect complement-fixing antigen for rubella virus in unconcentrated or 10-fold concentrated cell culture fluids(2, 4,6), we first looked for the antigen in the cellular phase of infected cell cultures. Infected RK-13 cells were harvested when the cultures showed 4-plus degeneration (8-10 days), and the cells were sedimented by centrifugation at 2000 rpm for 20 minutes. After removal of the supernatant fluid the cells were resuspended in 1/50th of the original volume of fluid and subjected to 3 cycles of freezing and thawing. In Table I it is seen that the unconcentrated, whole culture mate-

rial showed no complement-fixing activity with immune rabbit serum, and the cellular phase of the culture concentrated 50-fold showed specific fixation only when tested undiluted.

In successive experiments the fluid phase of infected cultures was collected after sedimentation of the cells and concentrated 50-fold by dialysis against polyethylene glycol. As shown in Table I, the fluid concentrate had a specific complement-fixing titer of 1:4 as compared to a titer of 1:1 for the cellular phase of the culture. An infected fluid concentrated 100-fold gave a titer of 1:8, while the cellular phase of the culture showed a titer of 1:1. Identical antigen titers were obtained in tests with a convalescent-phase serum from a known case of rubella.

Consequently, the antigens used for the studies described herein were prepared from the fluid phase of infected cell cultures, and concentrated by dialysis against polyethylene glycol.

Investigations on development of CF antigen in infected RK-13 cell cultures. Sever *et al*(9) have recently described a rubella complement-fixing antigen derived from infected cells harvested from cultures soon after inoculation with a massive dose of virus. Since we were more successful in detecting complement-fixing antigen in infected fluids than in cells, additional studies were performed to elucidate the formation and release of antigen in infected cultures.

Cultures inoculated as described under *Materials and methods* were harvested at various intervals after infection and the cellular and fluid phases were separated. Fluids were concentrated 100-fold by dialysis against polyethylene glycol and the cells were resuspended in 1/100th the original volume of culture fluid and subjected to 3 cycles of freezing and thawing. Antigens used for these studies were not treated with organic solvents. Both the fluid and cellular antigens were assayed for complement-fixing activity in block titrations against convalescent-phase serum from a known case of rubella.

Results of a representative experiment are shown in Table II where it is seen that in cultures harvested at 4 days only a very low level of CF activity was demonstrable, and then only in the fluid phase of the cultures. At 6 days identical titers of CF antigen were seen in the fluid and cellular phases, but thereafter greater quantities of CF antigen were released into the fluid until at 10 days virtually all of the CF activity was seen in the fluid phase. It would thus appear that the CF antigen is released into the fluid phase of the culture relatively rapidly after its formation in the cells.

TABLE II. Distribution of Rubella Virus Complement-Fixing Antigen in Infected RK-13 Cell Cultures at Various Intervals After Infection.

Antigen	Complement fixing titer at 4 days	6 days	8 days	10 days
Infected culture fluids 100×	1:1	1:2	1:4	1:8
Infected cells in 1/100 orig vol	0	1:2	1:2	1:1

Effect of organic solvents on the CF antigen. Since the CF antigens were concentrated by dialysis against polyethylene glycol they also contained high concentrations of serum and other non-sedimentable materials from the host cell cultures. They were therefore cloudy and often somewhat anticomplementary. It was found that nonspecific host materials could be reduced and the antigens rendered less anticomplementary by treatment with either Genetron 113 or ether, as described in *Materials and methods*. The specific antigen titers were unchanged or slightly improved by treatment with organic solvents. A greater volume of the antigen was lost in Genetron treatment than in ether treatment, and ether treatment possessed the additional advantage of rendering the antigen noninfectious.

Size of the CF antigen in relation to the infectious viral particle. The rubella virus has been shown to be morphologically similar to the myxovirus(13), and the possibility was considered that the CF antigen might be a "soluble" one separable from the infectious viral particle. In several experiments, rubella CF antigens (100-fold concentrates of infected tissue culture fluids) were subjected to centrifugation at 30,000 rpm for 3 hours, and the pellets and supernatant fluids were as-

TABLE III. Demonstration of Soluble Nature of Rubella Virus Complement-Fixing Antigen.

Antigen	Complement-fixing titer	Infectivity titer*
Infected culture fluids, 100×	1:8	$10^{-2.75}$
Centrifuged 30,000 rpm 3 hr:		
Sediment†	<1:1	$10^{-3.00}$
Supernatant fluid	1:8	$<10^{-1.00}$
Infected culture fluids, 100×, ether-treated	1:8	—
Centrifuged 30,000 rpm 3 hr:		
Sediment†	1:1	—
Supernatant fluid	1:8	—

* TCD_{50} per 0.1 ml of fluid; titrated in primary cultures of grivet monkey kidney cells.
† Resuspended to original volume.

sayed for complement-fixing activity. In Table III it is seen that virtually all of the CF activity remained in the supernatant fluid in both the untreated and ether-treated antigen, while the infectivity (of the untreated antigen) was concentrated in the pellet. Thus, the CF activity of rubella virus appears attributable to a soluble antigen.

Reactivity of CF antigen with human sera. Sera from 2 groups of patients with clinical diagnoses of rubella virus infection were examined in our preliminary studies on reactivity of the rubella CF antigens. The first group consisted of military recruits who were involved in an outbreak of rubella at Fort Ord, Calif., and the second was comprised of civilian patients on whom physicians had submitted specimens to our diagnostic laboratory. Sera from these patients were examined in neutralization and complement fixation tests for rubella virus, and results obtained to date are summarized in Table IV.

TABLE IV. Reactivity of Rubella Virus Complement-Fixing Antigen with Sera from Patients with a Clinical Diagnosis of Rubella Infection.

Patient group	No. tested	No. of patients with ≥ 4-fold rises in rubella antibody by: Both neut and CF tests	CF test only	Neut test only	Neither test
Military recruits	33	21	2	6	4
Civlian patients	29	7	3	3	16
Total	62	28	5	9	20

A large proportion of the military recruits showed significant antibody increases by both tests; a few more significant antibody rises were detected by the neutralization test alone than by the CF test alone; and only 4 of the 33 patients failed to show significant antibody rises by either test. All of these patients had an acute-phase serum specimen collected on the first day after onset of illness, and the convalescent-phase sera were taken 2 weeks later.

The group of civilian patients did not represent individuals from a single outbreak, and specimen collection was not so ideal as it was for the military recruits. Over one-half of the patients in this group failed to show antibody increases by either technic, but the two tests were comparable in their ability to demonstrate significant rises in antibody level.

A comparison of CF and neutralizing antibody titers of individual serum specimens is given in Fig. 1. Neutralizing antibody was demonstrable in a fairly high number of acute-phase sera in the absence of detectable CF antibody, but only 2 acute-phase specimens contained CF antibody without detectable neutralizing antibody; this probably reflects an earlier development of neutralizing antibody than CF antibody. In general, convalescent-phase sera showed higher titers of neutralizing antibody than of CF antibody. Some of the convalescent-phase sera showed the presence of neutralizing antibody, and no CF antibody, but the reverse was not seen.

As a test of the specificity of the rubella CF antigen, paired sera were tested from patients who had shown significant rises in antibody titer to other viral antigens. In Table V it is seen that of 43 patients who had shown significant increases in antibody level to myxovirus antigens or *Mycoplasma pneumoniae* CF antigen, only a single patient showed a significant rise in antibody to the rubella CF antigen, and 2 showed stationary titers of rubella CF antibody. The former patient also showed a 4-fold increase in rubella virus neutralizing antibody, and the latter 2 patients also had stationary levels of rubella virus neutralizing antibody.

Antibody responses of a rhesus monkey ex-

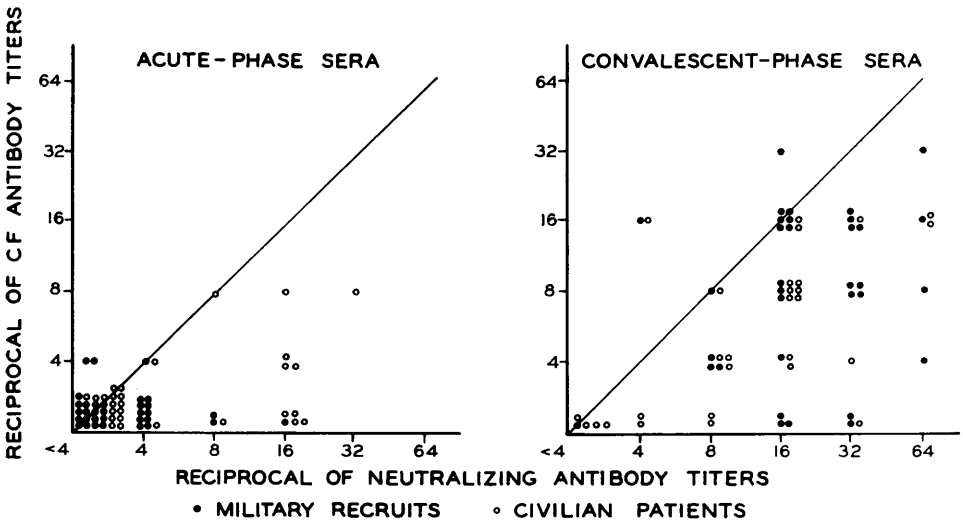

FIG. 1. A comparison of complement-fixing and neutralizing antibody titers of sera from patients with a clinical diagnosis of rubella.

perimentally infected with rubella virus. Wong and Belcourt(14), using a CF antigen derived from unconcentrated, infected primary rabbit kidney cell cultures and sera from grivet monkeys infected with rubella virus, found that monkey sera with high neutralizing antibody titers gave no reaction with the complement-fixing antigen, while sera with low or negative neutralizing antibody titers fixed complement in the presence of the antigen.

We have followed the antibody response of a monkey *(Macaca mulatta),* infected by intranasal instillation of rubella virus, through the incubation period and after the appearance of a rash, and the titers of sera collected at various intervals after infection are given in Table VI. Neither neutralizing nor CF antibody was demonstrable during the incubation period nor at the time of the appearance of a rash. A neutralizing antibody titer of 1:8 was detectable 1 week after the appearance of the rash, increasing to a titer of 1:32 at 3 weeks after the rash. Complement-fixing antibody appeared slightly later, at 2 weeks after the appearance of the rash. Thus, both types of antibody responses were elicited by the experimental infection, and titers increased over the course of the infection.

Discussion. The complement fixation test

TABLE V. Reactivity of Rubella Virus Complement-Fixing Antigen with Sera from Individuals Showing Significant Antibody Rises to Other Viral Antigens.

Antigen to which a ≧ 4-fold rise in titer detected*	No. of patients	CF reactions with rubella virus antigen: No antibody demonstrated (<1:4)	Stationary antibody titer	≧ 4-fold titer rise
Mycoplasma pneumoniae	5	3	1†	1‡
Rubella virus	5	5	0	0
Mumps virus	5	5	0	0
Influenza A virus	5	5	0	0
Resp syncytial virus	5	4	1†	0
Parainfluenza 1 virus	6	6	0	0
Parainfluenza 2 virus	6	6	0	0
Parainfluenza 3 virus	6	6	0	0
Totals	43	40	2	1

* CF antigens with the exception of parainfluenza virus types 1 and 3, which were hemagglutination antigens.

† Patient showed stationary neutralizing antibody titer for rubella.

‡ Patient showed 4-fold increase in neutralizing antibody titer for rubella.

TABLE VI. Antibody Responses of a Rhesus Monkey Experimentally Infected with Rubella Virus.

Antibody	Titers at indicated time after intranasal inoculation							
	Pre-inoc	10 days	14 days	21 days	27 days Rash	34 days	41 days	48 days
Neutralizing	<1:4	<1:4	<1:4	<1:4	<1:4	1:8	1:16	1:32
Complement-fixing	<1:4	<1:4	<1:4	<1:4	<1:4	<1:4	1:8	1:16

was found to be comparable to the neutralization test in its ability to detect significant rises in antibody titer in rubella virus infections. However, the complement fixation test was not as sensitive as the neutralization test for demonstrating antibody elicited by past infection. In general, CF antibody titers tended to be lower than neutralizing antibody titers, as has been found by Sever *et al*(9). Further investigations on complement-fixing antibody responses in rubella infections may reveal that any demonstrable level of CF antibody is good evidence of a recent infection. The complement fixation test possesses advantages over the neutralization test for diagnostic purposes in being less expensive and cumbersome to perform, less beset with variables, and the results are obtainable sooner.

Whether antigens prepared from the fluid phase of infected cell cultures as described herein or the cell-associated antigens described by Sever *et al*(9) are more satisfactory can be determined only by comparative testing. The antibody titers reported by the latter workers and those obtained with our antigens are similar. Although the cell-associated antigens may be somewhat easier to prepare, they require the use of very large viral inocula for infection of cell cultures, and the presence of cellular debris in the antigen may interfere with the reading of test results. Antigens derived from concentrated cell culture fluids and treated with ether are free from cellular debris, and ether treatment has been found to render the antigens noninfectious, a factor of some importance in their general use in diagnostic laboratories.

Although the results obtained in these studies are in general agreement with those obtained by Sever *et al*(9), they are at variance in several respects with those reported by Wong and Belcourt(14). The latter workers found unconcentrated, infected tissue culture fluids to possess high complement-fixing titers while we, as well as others(2,4,6) have been unable to demonstrate specific complement-fixing activity with unconcentrated tissue culture materials. Also, we found that, with two exceptions, complement-fixing antibodies did not occur in the absence of neutralizing antibody in sera from naturally-infected humans or an experimentally-infected monkey. (The two exceptions occurred in the case of 2 acute-phase human serum specimens which had CF antibody titers of 1:4 and neutralizing antibody titers of <1:4 to the rubella virus, but convalescent-phase sera showed high levels of both kinds of antibody.) Too, Wong and Belcourt reported that ether treatment inactivated the complement-fixing activity of their antigens, while we have found the activity of our antigens to be unchanged or slightly increased by ether treatment.

The finding that the complement-fixing activity of rubella virus is due to a soluble antigen separable from the infectious viral particle by centrifugation provides another example of the similarity of this virus to the myxoviruses.

Summary. A complement-fixing antigen for rubella virus has been produced by concentration of infected RK-13 cell culture fluids 100-fold by dialysis against polyethylene glycol. The antigen was shown to be separable from the intact viral particle by centrifugation (soluble antigen), and could be rendered non-infectious by ether treatment without undergoing a change in specific reactivity. The complement fixation test was found to be comparable to the neutralization test for de-

tecting significant increases in antibody level in rubella virus infections. Individuals showing significant rises in antibody titer to myxovirus antigens or *Mycoplasma pneumoniae* did not show antibody rises with the rubella complement-fixing antigen.

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Antibacterial Action of PMN Lysosomal Cationic Proteins Resolved By Density Gradient Electrophoresis.* (30749)

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Granules (lysosomes) from polymorphonuclear leucocytes (PMN) contain not only a variety of hydrolytic enzymes, but also highly cationic proteins that have antibacterial activity as well as the ability to produce inflammation and tissue injury(1,2,3,4).

Previous studies using paper electrophoresis and ethanol precipitation have shown that highly cationic proteins can be separated from the lysosomal enzyme activities(1). The ability of the cationic proteins to kill bacteria and invoke tissue damage may be a consequence of their strong electropositivity whereby they can interact with and disorganize membrane structure and destroy permeability barriers. They may also combine with and inactivate anionic constituents like nucleic acid, bacterial mucopolysaccharides and enzyme systems of the cells.

Because of the varied biological activities and electrophoretic heterogeneity of the cationic protein fraction, a preparative scale resolution of granule proteins by sucrose density gradient electrophoresis has been developed which provides further elucidation of lysosome constituents.

Materials and methods. A lysosomal fraction of rabbit peritoneal inflammatory PMN was prepared as previously described(1). The lysosome suspension was extracted with 0.01 N HCl and dialyzed against acetate buffer pH 4.0, ionic strength 0.01.

Ascending electrophoresis was carried out in a sucrose gradient column with acetate buffer pH 4.0, ionic strength 0.01, at 700

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