RABIES ANTIGENS RELEASED BY INFECTED CULTURES OF PRIMARY HAMSTER KIDNEY CELLS

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ABSTRACT

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The infectivity and immunogenicity of rabies virus in infected primary hamster kidney cell cultures was contained almost entirely in the cultural fluids rather than being cell associated. Antigens concentrated by filtration with a parlodion filter and then passed through a 0.05 μm pore diameter cellulose membrane filter contained 0%, 40%, and 45%, respectively, of the infective, complement-fixing, and immunogenic elements of the original cultural fluids. Virions were separated from noninfectious antigens by aminoethyl cellulose chromatography. The noninfectious "soluble" antigens were separated into at least 8 complement-fixing antigens by diethylaminoethyl- and ECTEOLA-cellulose chromatography.

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By

Roger Fredrick Everest

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DEDICATION

I dedicate this thesis to my wife, M. Louise Everest, and children, Roger, Jeannine, Craige, and Janet, without whose help and encouragement this work could not have been done.

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	3
Historical	3
Vaccines	3
Purification	4
Rabies virus	6
Soluble antigens	6
MATERIALS AND METHODS	9
Cell culture	9
Virus	10
Virus titration	10
Culture inoculation	10
Antigens	11
Viability test	11
Inactivation	12
Immunogenicity titration (vaccine potency test)	12
Complement-fixation (CF) titration	13
Antisera	14
Parlodion membrane filtration	14
Cellulose membrane filtration	14
Sephadex gel filtration	14
Ion exchange chromatography	15
RESULTS	17
Distribution of rabies virus and protective antigen in PHK cultures.	17
Appearance of CF antigen.	19
Antigen concentration.	19
Separation of noninfectious CF antigens and virus.	26
Infectivities, immunogenicities, and CF activities of pooled cultural fluids, concentrated antigens, and 0.05 μm pore diameter filtrates of concentrated antigens.	28
Exclusion filtration.	30
Chromatography of rabies antigens.	33
Chromatography of concentrated antigens.	35
Chromatography of 0.05 μm pore diameter filtrate.	41
Chromatography of dialyzed concentrated antigens.	41
Chromatography of concentrated antigens with four additional anion celluloses.	45

Chromatography of supernatant fluid and sediment from centrifuged rabies vaccine. 45

DISCUSSION 55

SUMMARY 58

BIBLIOGRAPHY 59

LIST OF TABLES

Table		Page
1.	Infectivity and immunogenicity of rabies-infected primary hamster kidney cells and cultural fluids	18
2.	Complement-fixation and infectivity of cultural fluids from rabies infected primary hamster kidney cell cultures	20
3.	Spearman rank correlation test of complement-fixation and infectivity titers of cultural fluids from rabies-infected primary hamster kidney cell cultures	21
4.	Preliminary studies of the effect of filtration of cultural fluids through parlodion membranes to reduce their volume and to concentrate the antigens retained by the membranes	23
5.	The effect of filtration of cultural fluids through parlodion membranes to reduce their volume and to concentrate the antigens retained by the membranes	25
6.	Separation of virions and noninfectious antigens by filtration of concentrated antigens through cellulose membrane filters	27
7.	Infectivity, immunogenicity, and complement-fixation of cultural fluids, concentrated antigens, and the 0.05 μm pore diameter filtrate of concentrated antigens	29
8.	Aminoethyl cellulose and ECTEOLA-cellulose chromatography of concentrated antigens	40

LIST OF FIGURES

Figure		Page
1.	Exclusion filtration through a 1 x 8.9 cm column of Sephadex G-200 of a 0.05 μm pore diameter membrane filtrate of concentrated antigens.	31
2.	Exclusion filtration through a 1 x 19.0 cm column of Sephadex G-200 of a 0.05 μm pore diameter membrane filtrate of concentrated antigens.	32
3.	Exclusion filtration through a 1 x 20.3 cm column of Sephadex G-200 of a 0.05 μm pore diameter membrane filtrate of concentrated antigens.	34
4.	Absorbancy at 254 nm of the effluent of concentrated antigens (Figure 5) following aminoethyl cellulose chromatography.	36
5.	Aminoethyl cellulose chromatography of concentrated antigens.	37
6.	ECTEOLA-cellulose chromatography of complement-fixing antigens not previously exchanged by aminoethyl cellulose from concentrated antigens (Figure 5).	39
7.	Aminoethyl cellulose chromatography of the 0.05 μm pore diameter membrane filtrate of concentrated antigens.	42
8.	Aminoethyl cellulose chromatography of previously dialyzed concentrated antigens.	44
9.	Aminoethyl cellulose chromatography of concentrated antigens diluted 10-fold.	46
10.	ECTEOLA-cellulose chromatography of concentrated antigens.	47
11.	Diethylaminoethyl cellulose chromatography of concentrated antigens.	49
12.	Aminoethyl cellulose chromatography of concentrated supernatant fluid following centrifugation of rabies vaccine.	50

13. Aminoethyl cellulose chromatography of sediment resuspended following centrifugation of rabies vaccine. 51

14. Diethylaminoethyl cellulose chromatography of concentrated supernatant fluid following centrifugation of rabies vaccine. 53

15. ECTEOLA-cellulose chromatography of concentrated supernatant fluid following centrifugation of rabies vaccine. 54

RABIES ANTIGENS RELEASED BY INFECTED CULTURES OF PRIMARY HAMSTER KIDNEY CELLS

INTRODUCTION

In some of our preliminary work (unpublished data), a consistent lack of correlation between infectivity and immunogenicity of rabies infected primary hamster kidney (PHK) cultures was found when the cultures were assayed at two day intervals following infection. The maximum level of infectivity was found either the 4th or 6th day post-infection (DPI) and the most immunogenicity, as determined by mouse potency tests, the 8th or 10th DPI. Furthermore, there was no correlation between infectivity of individual culture harvests and immunogenicity of vaccines made from them.

Supernatant fluids from centrifuged suspensions of infected mouse brains have been demonstrated to be immunogenic by Crick and Brown (1) and by Van den Ende, et al. (24). Although small residues of infectious virus were present, protective capacities of the fluids were greater than could be accounted for by the amounts of residual virus present. Crick and Brown (1) concluded that soluble antigens were contributing to the immunogenicities of the fluids. From these implications of soluble antigen immunogenicity and the report that two soluble antigens were demonstrated in fluid medium harvested from infected tissue cultures (14), we surmised that infected culture

fluids contained soluble immunogenic antigens. Immunogenic soluble antigens in medium harvested from our infected PHK cultures could account for lack of correlation between infectivity and protective potency.

The purpose of this work was to study rabies antigens in infected PHK cell cultures. To initiate the study, the distribution of optimal quantities of representative antigens in infected cultures was determined. Having located and obtained these antigens, their concentration was investigated.

The presence of small quantities of virus have interferred with prior studies to determine immunogenicity of soluble antigens. Elimination of this problem was studied using cellulose acetate membrane filtration to completely separate virions from noninfectious antigens without destroying immunogenicity of filterable, noninfectious antigens.

Also, anion exchange cellulose chromatography, utilizing diethylaminoethyl (DEAE), ECTEOLA, aminoethyl (AE), polyethyleneimine (PEI), and paraaminobenzyl (PAB) cellulose derivatives, was examined as a mechanism for separating and comparing antigens contained in a concentrated antigen preparation, cellulose acetate membrane filtrate of concentrated antigens, and resuspended sediment and supernatant fluid from centrifuged rabies vaccine.

LITERATURE REVIEW

Historical

The early information about rabies and its causative agent was reviewed by Johnson in Viral and Rickettsial Diseases of Man (7). A few facts from this review are cited in the following paragraphs.

Rabies was described by Democritis (500 B.C.) and Aristotle (322 B.C.). The relationship of hydrophobia in man to rabies in animals was recognized by Celsus (A.D. 100). The report by Zinke in 1804 of the transmission of rabies from a rabid to a normal dog by inoculation of saliva demonstrated the infectiousness of the disease. Passage of the infectious agent, by Remlinger (1903), through filters impervious to bacteria, established the ultramicroscopic nature of the virus.

Pasteur (1884) modified the pathogenicity of the virus by intracerebral passage in rabbits. Dogs immunized with a vaccine prepared from neural tissue containing modified, "fixed", virus were shown to be resistant to infection with natural virus. This type of vaccine was later utilized by Pasteur to treat persons exposed to rabies infection.

Vaccines

Modified Pasteur-type vaccines are still used in post-exposure treatment for rabies. The two vaccines generally used in the United

States are crude suspensions of animal tissues containing inactivated "fixed" virus. Semple vaccine (19) is a suspension of rabbit brain tissue containing virus which has been inactivated with phenol, and duck embryo vaccine (17) is a suspension of embryonic duck tissue containing virus which has been inactivated with beta propiolactone (BPL).

Purification

Immunization with these crude vaccines containing large amounts of extraneous tissue has produced serious allergic reactions (3, 10). In efforts to improve the vaccine and reduce painful and sometimes dangerous reactions to accompanying animal tissue, many attempts have been made to separate infectious virus from the tissue components.

Muller (12) applied suspensions of infected mouse brains to columns of Amberlite cation exchange resin. The resin removed some tissue components from fluids passing through the columns. Treated fluids retained 60% of the original virus infectivity and 20% of the original total nitrogen.

Purification using methanol precipitation was compared by Tagaya, Ozawa, and Kondo (22) to the use of pH 4.6 acid precipitation and to the use of 0.5% protamine. Methanol precipitation resulted in a recovery of 10% of infectivity and a removal of 87-88% of total nitrogen. Acid precipitation retained 10% of infectivity and removed 74% to 93% of total nitrogen. Less than 0.1% of infectivity was recovered by protamine purification (no data concerning the fate of accompanying nitrogen was provided).

Hottle and Peers (4) compared the effect of grinding infected rabbit brain tissue in distilled water to grinding it in the customary saline. Centrifugation at 1,000 x g for 1 hour of suspensions containing as little as 0.05 M NaCl sedimented 75% of protective potency along with much of the tissue debris. Supernatant fluids from centrifugation at 1,000 x g of a distilled water suspension retained 100% of protective potency and approximately 70% of total solids. Centrifugation of this supernatant fluid at 16,000 x g for 1 hour sedimented 108% of protective potency and 30% of total solids. The 16,000 x g sediment only produced brain lesions in 1 of 7 guinea pigs tested compared to lesions in 50% of animals tested with whole suspensions.

Purification by chromatography with ion exchange celluloses was examined by Thomas, et al. (23). Carboxymethyl (CM), a weak acidic cation exchange cellulose, did not exchange virus from infected mouse brain suspensions. Diethylaminoethyl (DEAE), a strong basic anion exchange cellulose, exchanged virus from infected suspensions, but only 1% of the infectivity was recovered in the column eluate. Chromatography with ECTEOLA, an intermediate basic anion exchange cellulose, exchanged virus from infected suspensions. Most tissue components passed through ECTEOLA-cellulose columns without being retained. Virus retained by the columns was eluted with 0.3 M KCl. The eluates contained as much infectivity as crude suspensions and protein nitrogen was reduced from 100 µg/ml to less than 5 µg/ml.

These methods did not yield protective antigen in sufficient quantities or in sufficient purity to be used for vaccine production.

However, Kissling's (8) finding that rabies virus was replicated in cultures of primary hamster kidney (PHK) cells offered a method of producing protective antigen free of potentially dangerous animal brain antigens. Following this report, replication of rabies virus was described in several other tissue culture systems (2, 5, 25, 27).

Rabies virus

The virus particle has been described as being bullet-shaped, with a hollow core, and having an attached thin-walled vesicle (15, 21). The particle, 75-100 nm in diameter and 125-180 nm long, is covered with protruding cylindrical subunits and contains a helical nucleo-capsid in the hollow core. The virus particle is the infectious agent and will agglutinate red blood cells and fix complement (13, 21). The virus also has ability to elicit neutralizing antibodies in immunized animals and a correlation between neutralizing antibody level and resistance to infection has been described (1).

Soluble antigens

"Soluble" antigen is a hazy term for noninfective, precipitating, complement-fixing (CF) antigen. It is a small, disease specific, particle, sedimenting more slowly than infective virus during centrifugation. The first report of rabies soluble antigen was made by Polson and Wessels (16). They emulsified rabies infected baby mouse brains in saline containing 10% inactivated rabbit serum. The emulsion was clarified by centrifugation for 60 minutes at 2500 rpm. The supernatant fluid was dialyzed for 48 hours, and then centrifuged at 30,000 rpm for 60 minutes to remove all infective virus. Centrifugation of

these extracts at different rotor velocities indicated that after removal of infectious virus there was little further reduction in CF titer. By a method of diffusion, coupled with CF tests, a rabies soluble antigen was determined to have a diameter of 12.4 nm.

Van den Ende, et al. (24) found soluble material in similar supernatant fluid had more CF activity than sedimented virus. Some of their soluble preparations had infectious titers of $1 \times 10^{1.0}$ and CF titers of 1/1600. Preparations which did not contain detectable virus had scanty neutralizing activity. A single soluble antigen was detected by electrophoresis of these preparations.

Following extensive purification, Meade (11) identified two soluble antigens in infected suckling mouse brain emulsions and characterized them by sedimentation rates, behavior towards trypsin, and immunodiffusion in gel. However, the antigenicity of these preparations, aside from their CF capacity, was not reported.

Crick and Brown (1) centrifuged infected suckling mouse brain emulsions at 15,000 rpm for 2 hours and assayed the top 9 ml and the bottom 2 ml of the supernatant fluid as well as the pellet for infectivity and for ability to elicit neutralizing antibody in adult mice. The immunizing capacities of the supernatant fractions were greater than was expected from their infectious titers and, therefore, Crick and Brown (1) concluded that soluble antigens were contributing to the immunogenicity.

The presence of two soluble antigens in medium from infected hamster kidney fibroblast, BHK 21/C13, cultures was demonstrated by

density gradient centrifugation (14). Antigens were precipitated from culture medium with zinc acetate and centrifuged at 49,500 x g for 4 hours to remove virus. Supernatant material was centrifuged to equilibrium in cesium chloride density gradients. A single band of activity, with a density of 1.26 g/ml, was detected by immunofluorescent assay (14). The same supernatant fluid was applied to preformed sucrose gradients and centrifuged at 124,000 x g for 270 minutes. Two bands of activity having sedimentation coefficients of 10 S and 23 S were found.

MATERIALS AND METHODS

Cell culture. Kidneys removed aseptically from 10 to 14 exsanguinated hamsters were minced with scissors and placed in a 500 ml trypsinizing flask containing 100 ml of 0.25% trypsin (Difco Laboratories, Inc., Detroit, Mich.) in Hanks' balanced salt solution. The solution was stirred gently, but briskly, for 30 minutes with a magnetic stirrer and wash liquid removed from the flask and discarded. Fresh trypsin solution was placed in the flask and stirring continued until liquid turbidity indicated the presence of large numbers of suspended cells. Fluid and cells were removed from the flask and centrifuged at 50 x g for 20 minutes. Sedimented cells were resuspended in 10 ml of growth medium consisting of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution [(HLH) (Grand Island Biological Co., Grand Island, N.Y.)] containing 10% fetal calf serum (fcs). This procedure was repeated 8 to 10 times, until very few cells were freed from the tissue. A small sample of resuspended cells was mixed with an equal volume of trypan blue and the number of unstained viable cells/ml, usually 1-3 million, determined using a hemocytometer.

Volume of the cell suspension was adjusted with HLH to contain 800,000 viable cells/ml and placed in culture bottles: 15 ml into 8 oz. milk dilution, 200 ml into 2 l Povitsky, and 400 ml into 5 l

Povitsky bottles. After incubation of the cultures at 37°C for 4 days, spent medium was decanted and replaced with fresh medium, and incubation continued until growing cells covered the bottom surfaces of the culture bottles, usually requiring 3 to 4 days.

Virus. The rabies virus used for these studies was the tissue culture adapted strain of CVS-11 received from Dr. Kissling of the National Communicable Disease Center, Atlanta, Georgia.

Virus titration. Rabies virus titrations were carried out with serial 1 ml ten-fold dilutions of virus in distilled water containing 2% normal horse serum. At least four dilutions were tested, using five 3-week-old mice for each dilution. Mice were inoculated intracerebrally with 0.03 ml of the test dilution.

All mice were observed for 14 days from the time of inoculation. Only those deaths occurring after the 5th day and preceded by signs of fixed rabies (paralysis, convulsions) were considered rabies deaths. Any mice becoming paralyzed, but surviving the 14-day observation period, were considered the same as deaths due to rabies. Titers were calculated by the Reed-Muench method (18) and expressed as LD_{50}/.03 ml.

Culture inoculation. Spent medium was decanted from monolayered PHK cultures and the cells inoculated with viral suspensions, diluted with distilled water to contain approximately 1 million LD_{50}/ml, in a volume one-tenth the total volume of medium. Inoculated cultures were incubated for 1 hour at 37°C and agitated every 10 minutes. The viral suspensions were decanted, the original volume of medium restored with HLH containing 3% fcs, and the cultures incubated at 35°C.

Antigens. For determining distribution of antigens in the first part of the study, spent medium was renewed on the 2nd and 5th DPI. On the 10th DPI fluid medium was decanted from the cultures, designated as decanted fluid, and replaced with an equal volume of fresh medium. Following one freeze-thaw cycle, the fresh medium and suspended cells were removed from the bottles, ground for three minutes in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.), and designated as cell suspension.

Infectious fluids were also obtained from infected PHK cultures by renewing the medium each 48 hours and collecting the fluids decanted on the 2nd, 4th, 6th, 8th, and 10th DPI.

Materials similar to historical "soluble" (16) and sedimented viral antigens were obtained from the Michigan Department of Public Health viral vaccine production unit. These antigens had been separated by centrifuging (125,000 x g for 3 hours) inactivated cultural fluids. Supernatant fluid from the centrifugation was passed through a sequence of treated membrane filters, terminating with a 0.05 μm pore diameter membrane to remove any virus which might be present. The 0.05 μm pore diameter filtrate retained all the CF activity of the original fluid. Sediment from the centrifugation was resuspended to 1/100 of original volume in Parker's 199 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 0.25% human serum albumin.

Viability test. The absence of viable rabies virus was determined by inoculating five mice intracerebrally with 0.03 ml of undiluted or 10-fold concentrated materials. The mice were observed 14 days for symptoms of rabies.

Inactivation. Infectious materials used for immunogenicity titrations were inactivated with beta propiolactone (BPL) (Fellows-Testagar Division, Fellows Medical Manufacturing Co., Inc., Detroit, Mich.) . A 1% solution of BPL, prepared in 2° ± 2°C distilled water, was added to the infectious material to a final concentration of 0.02% (V/V). The solutions were incubated 2 hours at 37°C and for an additional 24 hours at 4°C. The resulting vaccines were tested for presence of viable virus.

Immunogenicity titration (vaccine potency test). Vaccine potency was measured by the standard National Institutes of Health rabies vaccine potency test (20). The results were calculated by the Reed-Muench method, and expressed as the reciprocal of the dilution of vaccine which would protect 50% of vaccinated mice (ED_{50}) when challenged by 5-50 LD_{50} of virus. Four 5-fold dilutions of each material tested were prepared in buffered saline solution (0.85% NaCl in 0.02 M phosphate-buffer solution, pH 7.6). Each of ten mice, 11-15 g, was injected intraperitoneally with 0.5 ml of a single dilution of vaccine. Two doses of vaccine were given to each mouse one week apart. Enough mice were set aside at the time of the first vaccination for a titration of challenge virus.

Immunity of vaccinated mice was challenged 14 days after the first vaccination. Challenge virus (CVS 27) was diluted in distilled water containing 2% normal horse serum to contain between 5 and 50 LD_{50} of virus, and each mouse was innoculated intracerebrally with 0.03 ml of viral suspension.

Challenge virus was titrated in control mice using five mice/dilution and four 10-fold dilutions commencing with the dilution used to challenge vaccinated mice.

Mice were observed for 14 days for symptoms of rabies. Deaths occurring after the 3rd day and those preceded by signs of fixed virus rabies were considered deaths from rabies.

Complement-fixation (CF) titration. Complement fixation testing was performed according to the Laboratory Branch Complement Fixation Method (LBCF) of the Public Health Service (9). Antigens were serially diluted 1/2 in veronal buffered gelatin solution (VBD) (9). A 0.2 ml volume of each antigen dilution was incubated overnight at 4°C in a 1 x 7.5 cm tube along with 0.2 ml of hamster antirabies serum and 5 units of guinea pig complement diluted with VBD to 0.4 ml. After overnight incubation, 0.2 ml of sheep red blood cells (RBC), sensitized with hemolysin, was added to each tube and the tubes incubated for 1 hour at 37°C. The amount of hemolysis was determined by comparison with fresh color standards, ranging from 0 to 100% hemolysis, prepared in the same volume (1 ml) from the same preparation of sheep RBC. The CF titer was designated as the reciprocal of the highest antigen dilution which demonstrated 30% or less hemolysis.

The LBCF method was modified for unconcentrated antigen preparations and for fractions collected from Sephadex and ion exchange cellulose columns. Complement-fixing ability of these materials was expressed as percent hemolysis obtained with a 2-fold dilution of the material in VBD, instead of titer.

Antisera. Rabies antisera were obtained from blood of hamsters immunized with homogenized rabid hamster brains as described by Johnson (6).

Parlodion membrane filtration. Infectious culture media harvested by decantation on the 2nd, 4th, 6th, 8th, and 10th DPI were clarified by filtration through a 0.45 μm pore diameter cellulose membrane (Millipore Corp., Bedford, Mass.), and concentrated by filtration, under vacuum, with 7% parlodion-coated 4.7 x 13 cm alundum thimbles, according to the procedure used by the Michigan Department of Public Health, Bacterial Vaccine Section, for concentrating toxins. Thimble leakage was monitored by testing filtrates with 30% tricloroacetic acid and by mouse innocuity tests. Concentrated 6, 8, and 10-day harvests were pooled, designated as concentrated antigens, and stored at -20°C. Volumes of less than 500 ml were concentrated by the same method, using parlodion coated 2.5 x 7.2 cm alundum thimbles.

Cellulose membrane filtration. Fetal calf serum, diluted to 10% with distilled water was clarified by filtration in sequence through 0.45, 0.22, 0.10, and 0.05 μm pore diameter membrane filters (Millipore Corporation, Bedford, Mass.). One hundred ml of the clarified serum, 30 ml of 0.15 M phosphate buffer, pH 7.2, and the concentrated fluids, in that order, were filtered in sequence through 47 mm diameter membrane filters of 0.45, 0.22, 0.10, and 0.05 μm pore diameter.

Sephadex gel filtration. Ten grams of Sephadex G-200, 40-120 mesh (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), were placed in an excess of 0.1 M phosphate buffer, pH 7.5, and allowed to swell

at room temperature for 5 days. The swollen gel was carefully poured into glass columns 0.8 cm in diameter to form beds 8.9, 19.0, and 20.3 cm in height, respectively.

Each gel column was equilibrated with 100 ml of 0.1 M phosphate buffer, pH 7.5. One milliliter aliquots of 0.05 μm pore diameter filtrate were applied to the columns and eluted with 0.1 M phosphate buffer, pH 7.5. Filtrates were collected manually in 12-drop fractions.

Ion exchange chromatography. Diethylaminoethyl (DEAE), ECTEOLA, aminoethyl (AE), polyethyleneimine (PEI), and paraaminobenzyl (PAB) cellulose derivatives (Bio-Rad Laboratories, Richmond, Calif.) were used in this study. Five grams of dry cellulose derivative was stirred with 400 ml of 0.01 M tris (hydroxymethyl aminomethane)- HCl (tris) buffer, pH 7.3, for 30 minutes, allowed to settle for 15 minutes, and the small suspended cellulose particles (fines) decanted. This procedure was repeated until most of the "fines" were removed. The cellulose was then suspended in 150 ml 0.01 M tris buffer and the suspension adjusted to pH 7.3.

Chromatographic columns of 1 cm diameter and 8 to 9 cm in height were prepared from 40 ml of cellulose suspension. After settling, the cellulose bed was washed with 500 ml of 0.01 M tris buffer, pH 7.3, containing NaCl of a molarity equivalent to the chloride ion concentration (Cl^-) of the sample to be applied.

Following application of a sample, the ion exchange medium was washed free of nonexchanged materials with 0.01 M tris buffer, pH 7.3, containing NaCl equivalent to the (Cl^-) of the sample. Elution of exchanged materials was carried out stepwise with increasing

concentrations of NaCl in 0.01 M tris buffer. The column effluent was collected in 2 ml aliquots by a volumetric fractionator (Model V-10, Gilson Medical Electronics, Middleton, Wisc.). The absorbancy of the effluent was monitored at 254 nm wavelengths by an ultraviolet analyzer (Model VA-2, Instrument Specialties Co., Inc., Lincoln, Neb.). Elution at each NaCl concentration level was continued until the effluent had no absorbancy at 254 nm.

RESULTS

Distribution of rabies virus and protective antigen in PHK cultures. The fluid medium and cells of rabies-infected cell cultures were examined to determine the distribution of virus and protective antigen.

Following medium renewal on the second and fifth days, the fluid medium was decanted from the cells on the tenth day postinoculation and replaced with fresh medium. The fresh medium and cells were frozen and thawed once, removed from the bottles, and ground for three minutes in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.). Both decanted fluids and homogenized cellular suspensions were tested quantitatively for infectious virus and immunogenicity (protective antigen).

Each decanted medium contained more than 3,900 mouse LD_{50} of infectious virus/0.03 ml. In contrast, the cellular suspensions contained less than 100 LD_{50}. Furthermore, the decanted fluids had protective potency EDs_{50} of 44, 60, and 67, while the cellular suspensions EDs_{50} were 10, < 5, and < 5 (Table 1).

These findings indicated that infected cells released most of the virus and protective antigen to the fluid medium. Consequently, in subsequent experiments only decanted infectious fluids were investigated.

TABLE 1. Infectivity and immunogenicity of rabies-infected primary hamster kidney cells and cultural fluids

Trial	Cultural fluids		Cells	
	Infectivity[a]	Protective Potency[b]	Infectivity	Protective Potency
1	3.8	44	< 2.0	10
2	3.6	67	< 2.0	< 5
3	3.6	60	< 2.0	< 5

[a] Log_{10}, LD_{50}/.03 ml.

[b] ED_{50}/ml.

Appearance of CF antigen. In previous work maximal levels of infectious virus and protective antigen were present in infected cell cultures at different postinoculation periods (unpublished data). This suggested that, to obtain sufficient quantities of CF antigen for study, cultures should be tested to determine the optimal harvest time for CF antigens, since it could not be assumed that the maximum CF titer would correlate with either the maximum infectivity or immunogenicity.

Fluids from four infected and four noninfected control cultures were harvested at 2, 4, 6, 8, and 10 DPI and tested individually for infectivity and complement-fixation. Phenol red in the fluids obscured hemolysis, therefore, the samples were diluted 2-fold with VBD for testing. Most of the infected fluids did not have sufficient CF to produce 30% hemolysis and none of the control fluids had any CF activity (Table 2).

Correlation of CF and infectivity titers as the mean of 5 observations was examined by the Spearman rank correlation test (Table 3). The correlation coefficient (r_s) of 0.80 indicated that there was no correlation of the maxima at the 0.05% level of significance.

Trial 4 was initiated with the intention of utilizing the large volumes of cultural fluids for the remainder of the study. However, the CF levels were too low to be used for monitoring noninfectious antigens, therefore, concentration of the harvests was undertaken.

Antigen concentration. Filtration with alundum thimbles coated with 7% parlodion, which retains substances having molecular weights

TABLE 2. Complement-fixation and infectivity of cultural fluids from rabies infected primary hamster kidney cell cultures

Trial	Titration	Days post-inoculation				
		2	4	6	8	10
1	CF[a]	60	45	35	30[c]	45
	Infectivity[b]	< 2.0	3.83	4.5	3.75	< 2.0
2	CF	55	35	35	45	55
	Infectivity	4.17	5.30	4.46	4.22	3.17
3	CF	50	35	25	35	50
	Infectivity	3.17	4.38	4.38	3.5	3.17
4	CF	80	10	55	45	80
	Infectivity	3.68	3.82	4.17	3.17	2.75
Mean titer	CF	61.25	31.25	37.50	38.75	57.50
	Infectivity	3.26	4.33	4.38	3.66	2.77

[a] Percentage hemolysis of a 1/2 dilution.

[b] Log_{10}, LD_{50}/.03 ml.

[c] Numbers underlined are the highest titration of each trial.

TABLE 3. Spearman rank correlation test of complement-fixation and infectivity titers of cultural fluids from rabies-infected primary hamster kidney cell cultures

Day postinoculation	Rank of mean titers		Difference (d)	
	CF	Infectivity	d	d^2
2	4	5	1	1
4	2	1	1	1
6	1	2	1	1
8	3	3	0	0
10	5	4	1	1
				Sum of d^2 = 4

Correlation coefficient: $r_s = 1 - \frac{6 \quad d^2}{N3 - N}$

$= 1 - \frac{6\ (4)}{125-5}$

$= 0.80$

greater than 70,000, is used by the Michigan Department of Public Health Laboratories for concentrating toxoids.

Six trials were made to test the efficacy of this procedure in concentrating infectious fluids. Two hundred milliliters were concentrated with each filtration.

Fluid was not agitated during the first trial and at its termination the thimble was removed from the concentrate. The concentrate retained only 2.5% of CF activity and 2.1% of infectivity, but the filtrate also retained 0.2% of the infectivity (Table 4). This was not only a dismal failure in concentrating antigens, but even worse, original activities were not recovered. One explanation for this failure would be the attachment of antigens to parlodion and their removal along with the thimble.

In an attempt to verify this hypothesis, a second trial was made. Fluid was not agitated during the second trial, however, at its termination the surface of the thimble was washed by vigorously pipetting concentrate over it. As a result of a 10-fold reduction in volume, CF activity was increased 8-fold and infectivity 7.1-fold. However, the filtrate again contained 0.2% of original infectivity.

This procedure was repeated for the third trial and results were similar to those obtained with the second trial except infectivity of the filtrate, $< 0.1\%$, was below the level tested.

Normal fluids harvested from noninoculated PHK control cultures were concentrated in the fourth trial. Infectivity of this material was not tested, but, since the concentrate was prepared as a normal CF control, CF activity of the initial fluid and concentrate was

TABLE 4. Preliminary studies of the effect of filtration of cultural fluids through parlodion membranes to reduce their volume and to concentrate the antigens retained by the membranes

Filtrations	Cultural fluids		Filtrate	Concentrated antigens		Cultural fluids ml / Concentrated antigens ml
	Infectivity[a]	CF[b]	Infectivity	Infectivity	CF	
1[c]	4.17	4	1.38	3.50	1	10
2[d]	4.17	4	1.32	5.02	32	10
3[d]	3.63	4	0	4.5	32	10
4[d,e]	....	0	....	....	0	10
5[f]	4.5	4	0	5.38	64	10
6[f]	3.5	4	0	4.8	32	13

[a] Log_{10}, LD_{50}/.03 ml.

[b] Reciprocal of the highest dilution of antigen producing 30% or less hemolysis.

[c] Cultural fluids not stirred and filter surface not washed.

[d] Cultural fluids not stirred, filter surface washed.

[e] Cultural fluids from normal, uninoculated primary hamster kidney cell cultures.

[f] Cultural fluids stirred, filter surface not washed.

tested. These fluids were not anticomplementary and did not fix complement in the presence of anti-rabies serum.

In attempts to prevent antigen attachment to parlodion, fluids were briskly stirred by a magnetic stirrer during the fifth and sixth trials. At their termination thimbles were not washed before being removed from the concentrates. Results were similar to those with the third trial, thus further supporting the validity of the original hypothesis. The loss of antigens with the first trial was due to their attachment to parlodion.

Following the demonstration that filtration adequately concentrated infectivity and CF activity, cultural fluids were concentrated as follows: fluids were stirred during concentration, and thimbles were not washed at the termination. Two liters of fluids harvested at 2, 4, 6, 8, and 10-days postinoculation, and a pool of the 2, 4, 6, 8, and 10-day fluids harvested from a normal culture were concentrated at least 10-fold.

Virus titers of the 2, 4, 6, and 8-day fluid harvests increased to values consistent with the decrease in volume (Table 5). Complement-fixing activity was demonstrated in all the concentrates prepared from infectious fluids. The 10-fold concentrate of pooled noninfectious culture fluids was not anticomplementary and did not fix complement in the presence of anti-rabies serum. The 6, 8, and 10-day concentrates of fluids decanted from infected cultures were pooled and used as concentrated antigens during the remainder of the study.

TABLE 5. The effect of filtration of cultural fluids through parlodion membranes to reduce their volume and to concentrate the antigens retained by the membranes

Day post-inoculation	Cultural fluids		Concentrated antigens		Cultural fluids ml / Concentrated antigens ml
	Infectivity[a]	CF[b]	Infectivity	CF	
2	3.63	< 2	4.83	2	12.5
4	4.5	4	5.38	16	10.0
6	3.5	< 2	4.83	8	11.0
8	3.17	< 2	4.55	4	10.5
10	< 2.0	< 2	2.38	4	10.0
Control	....	0	....	0	10.0

[a] Log_{10}, LD_{50}/.03 ml.

[b] Reciprocal of the highest dilution of antigen producing 30% or less hemolysis.

Separation of noninfectious CF antigens and virus. Apparently there is a difference between the size of rabies virus and soluble antigens. Rabies virus has been reported to measure 100 x 140 nm (15) and to have a sedimentation coefficient of 600 S (14). In contrast, one soluble antigen was calculated to be 12 nm in diameter (16), and two antigens were calculated to have sedimentation coefficients of 10 S and 23 S (14).

An attempt was made to take advantage of this difference in size to separate soluble antigens from virus by filtration. A trial filtration of 50 ml of concentrated antigens was made through membrane filters treated with fcs, according to the procedure described by Ver, et al. (26). Results of this first trial (Table 6) made a second separation mandatory, not only to confirm the first results, but also to obtain a larger volume of filtrate for further study.

Infectious virus passed through 0.45, 0.22, and 0.10 μm pore diameter filters, but was retained by the 0.05 μm pore diameter filter (Table 6). In the first trial and in the second trial 99.97% of infectivity was recovered. Infectious virus was not found in 0.05 μm pore diameter filtrates of either trial, even though the filtrates were concentrated 10-fold by ultrafiltration.

Results of CF tests were identical for both trials. All CF activity passed through the first three filters and was recovered in the filtrates. The 0.05 μm pore diameter filters retained 50% of CF activity, along with virus, but the other 50% of CF activity was recovered in the noninfectious filtrate (Table 6).

TABLE 6. Separation of virions and noninfectious antigens by filtration of concentrated antigens through cellulose membrane filters

	First test		Second test	
	Infectivity[a]	CF[b]	Infectivity	CF
Concentrated antigens	3.5	8	3.83	8
0.45 μm pore diameter filtrate	3.38	8	3.63	8
0.22 μm pore diameter filtrate	3.5	8	3.68	8
0.10 μm pore diameter filtrate	3.22	8	3.5	8
0.05 μm pore diameter filtrate	noninfectious	4	noninfectious	4

[a] Log_{10}, LD_{50}/.03 ml.

[b] Reciprocal of the highest dilution of antigen producing 30% or less hemolysis.

Immunogenicity of the virus-free 0.05 μm pore diameter filtrate was examined by inoculating five mice with undiluted filtrate and subsequently challenging with 10 LD_{50} of CVS virus. None of the mice died or showed any symptoms of rabies. This ability of virus-free filtrate to protect mice against a rabies virus challenge confirmed the assumption that noninfectious antigens were immunogenic.

Infectivities, immunogenicities, and CF activities of pooled cultural fluids, concentrated antigens, and 0.05 μm pore diameter filtrates of concentrated antigens. As a result of the demonstration that noninfectious antigens were immunogenic, infectivities, immunogenicities, and CF activities of infectious cultural fluids, concentrated antigens, and 0.05 μm pore diameter filtrates of concentrated antigens were compared. Equal volumes (approximately 67 ml) of 6, 8, and 10-day cultural fluids were pooled (200 ml) and concentrated 10-fold by filtration. Concentrated antigens were filtered through a sequence of treated cellulose membranes (0.45, 0.22, 0.10, and 0.05 μm pore diameter).

The concentrated antigens had 8-fold higher CF activity, 8.9-fold greater infectivity, and more than 3-fold greater immunogenicity (at a dilution of 1/125, eight of ten immunized mice showed no symptoms following challenge) than the initial pool of cultural fluids (Table 7). These increases in activity were accompanied by a 10-fold reduction in fluid volume.

Again, as in the previous experiment, the 0.05 μm pore diameter filtrate had no infectivity, even when tested at a 10-fold concentration,

TABLE 7. Infectivity, immunogenicity, and complement-fixation of cultural fluids, concentrated antigens, and the 0.05 μm pore diameter filtrate of concentrated antigens

	CF[a]	Infectivity[b]	Protective potency[c]	Relation to cultural fluids			
				Volume	CF	Infectivity	Protective potency
Cultural fluids	1	2.88	41	1	-	-	-
Concentrated antigens	8	3.83	> 125	10	8	8.9	> 3
0.05 μm pore diameter filtrate	4	0	187	10	4	0	4.6

[a] Reciprocal of the highest dilution of antigen producing 30% or less hemolysis.

[b] Log_{10}, LD_{50}/.03 ml.

[c] ED_{50}/ml.

and, again, 50% of the concentrated antigens CF activity was retained by the filter and 50% was recovered in the filtrate.

Recovery of 46% of initial protective potency in the 0.05 μm pore diameter filtrate along with 40% of initial CF activity and absence of infectious virus confirms the immunogenicity of noninfectious antigens.

Exclusion filtration. The possibility that 0.05 μm pore diameter filtrate contained antigens of dissimilar size was suggested by the reported difference in sedimentation rates (10 S and 23 S) of two soluble antigens demonstrated in fluid medium from rabies infected BHK 21/C13 cultures (14). Separation of soluble antigens based on their size difference was investigated utilizing exclusion filtration of 0.05 μm pore diameter filtrate through a 0.8 cm x 8.9 cm column of Sephadex G-200.

Effluent from the initial trial had considerable CF activity in fractions 1 and 2, followed by an extensive trailing edge of low CF activity (Figure 1). This indicated exclusion of one or more antigens in the large peak and retardation of smaller (below 800,000 M.W.) antigens in the trailing area. The void volume, which was discarded, contained some activity.

For the second filtration column length was increased to 19.0 cm to facilitate better separation. There was a single large CF peak in the early fractions (4 and 5) and also a small peak in the trailing edge (Figure 2). Although the two peaks were not completely separated, the minor peak indicated small antigens were present and true separation might be possible.

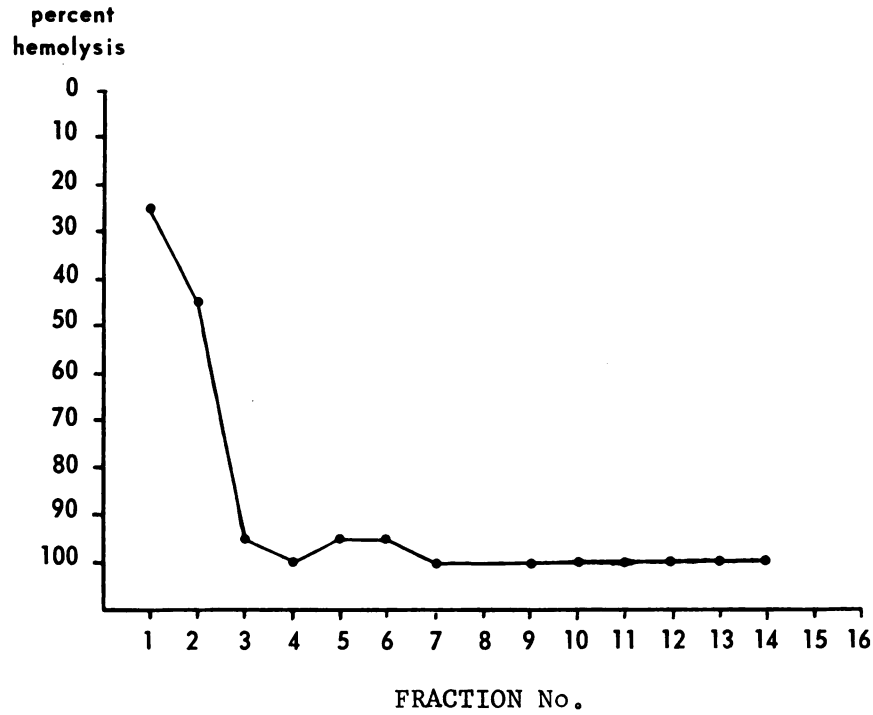

FIGURE 1. Exclusion filtration through a 1 x 8.9 cm column of Sephadex G-200 of a 0.05 μm pore diameter membrane filtrate of concentrated antigens.

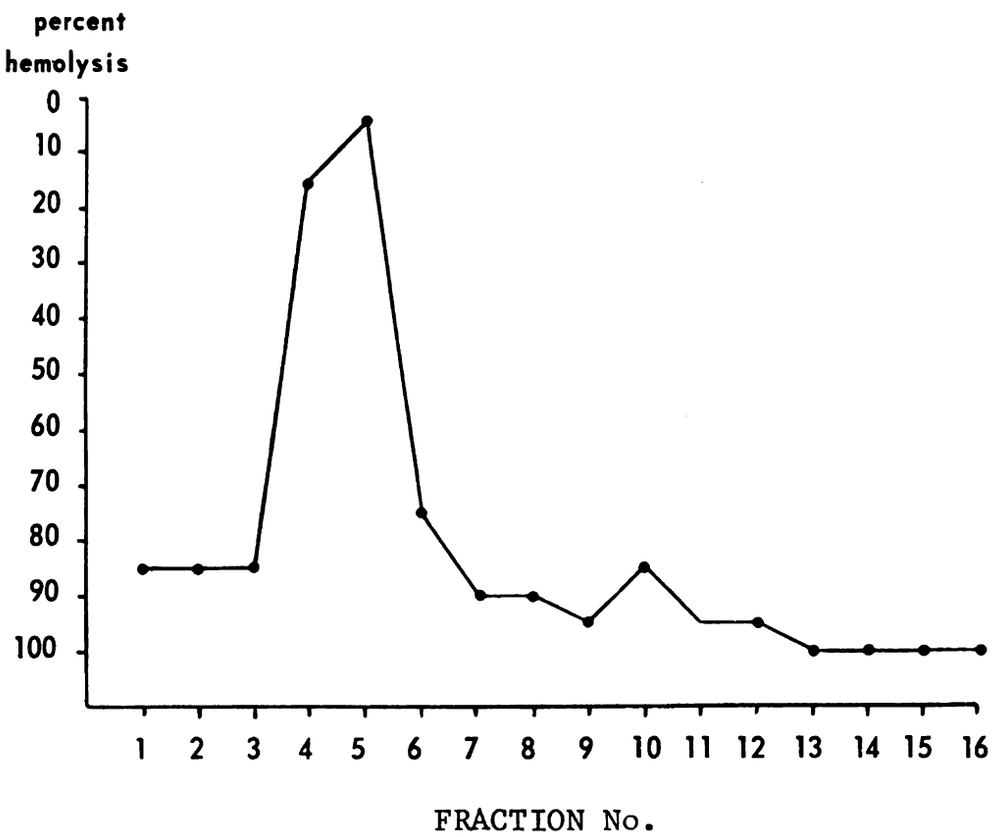

FIGURE 2. Exclusion filtration through a 1 x 19.0 cm column phadex G-200 of a 0.05 μm pore diameter membrane filtrate of ntrated antigens.

A third filtration was done to determine if the minor peak of the
nd filtration was obtained again and hence could be considered sig-
cant. Filtrate from this column contained a large area of activity
ne early fractions followed by a long, low trailing edge, without
inor peak (Figure 3).

Chromatography of rabies antigens. Thomas, et al. (23) found
DEAE-cellulose exchanged rabies virus from infected brain tissue
rial, but the infectivity was not recovered by elution. They were
to recover virus exchanged by less basic ECTEOLA-cellulose, how-
, the volume of eluent required to free all the infectious virus
ed the activity 25-fold.

These results implied that cellulose derivatives less basic than
LA would retain the antigens less tenaciously and allow them to
covered without dilution. Aminoethyl, a cellulose derivative
basic than ECTEOLA, was used in the first attempt of this investi-
n to separate and recover rabies antigens relatively undiluted.
During preliminary work, a small amount of CF activity, retained
-cellulose, was eluted by 0.1 M NaCl. Thereafter, fluids
M Cl^-) were usually diluted 2-fold with distilled water to allow
nge at 0.07 M Cl^- and elution with 0.1 M NaCl.

A continuously increasing concentration of NaCl was used as eluent
he preliminary cellulose chromatography. This method did not
CF activity in discrete portions. For this study, a discontin-
gradient of NaCl, increasing in concentration in a step wise
r, was used as eluent. Elution by each concentration of NaCl was

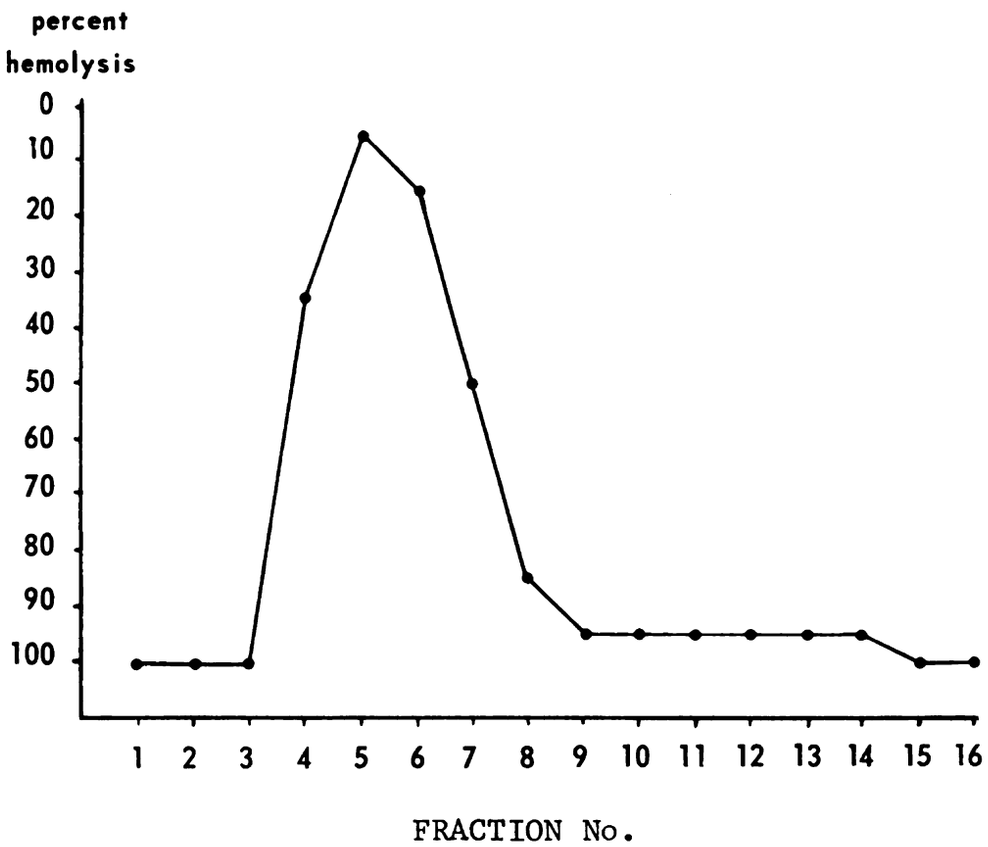

FIGURE 3. Exclusion filtration through a 1 x 20.3 cm column of ıdex G-200 of a 0.05 μm pore diameter membrane filtrate of concen-ɛd antigens.

nued until the eluate was washed from the column and the effluent
o adsorbancy at 254 nm. A more concentrated NaCl solution was
applied to the cellulose derivative (Figure 4).

Chromatography of concentrated antigens. Fifteen milliliters of
ntrated antigens were diluted with an equal volume of distilled
and chromatographed on AE-cellulose. A relatively large amount
activity was not retained (Figure 5). There was no CF activity
e 0.1 and the 0.3 M NaCl eluates. The 0.15, 0.20, and 0.25 M
eluates contained small amounts of activity and the 0.4 and
portion of the 0.5 M NaCl eluates were very active. The latter
on of the 0.5 and all the 0.75 M NaCl eluate had erratic CF
ity, typical of the materials containing a large amount of NaCl.
the CF active fractions of the 0.2, 0.25, 0.4, and 0.5 M NaCl
es contained infectious virus, these fractions were combined.
Fractions 7 through 18 contained CF antigens which were not
ned by AE-cellulose at 0.07 M Cl^-. Fractions 8 through 17
1) were pooled as being representative of the nonretained CF
ens. The CF activity of fractions 7 and 18 were so low it was
ed their inclusion would dilute the pool. The antigens not
ned were innocuous for mice and presumably represented "soluble",
fectious, CF antigens.

Eighteen milliliters of effluent containing the antigens not
ned by AE-cellulose were chromatographed on more basic ECTEOLA-
lose in an attempt to exchange and separate these antigens.
ame washing and elution procedures were used as were employed
AE-cellulose. The major portion of CF activity was not exchanged

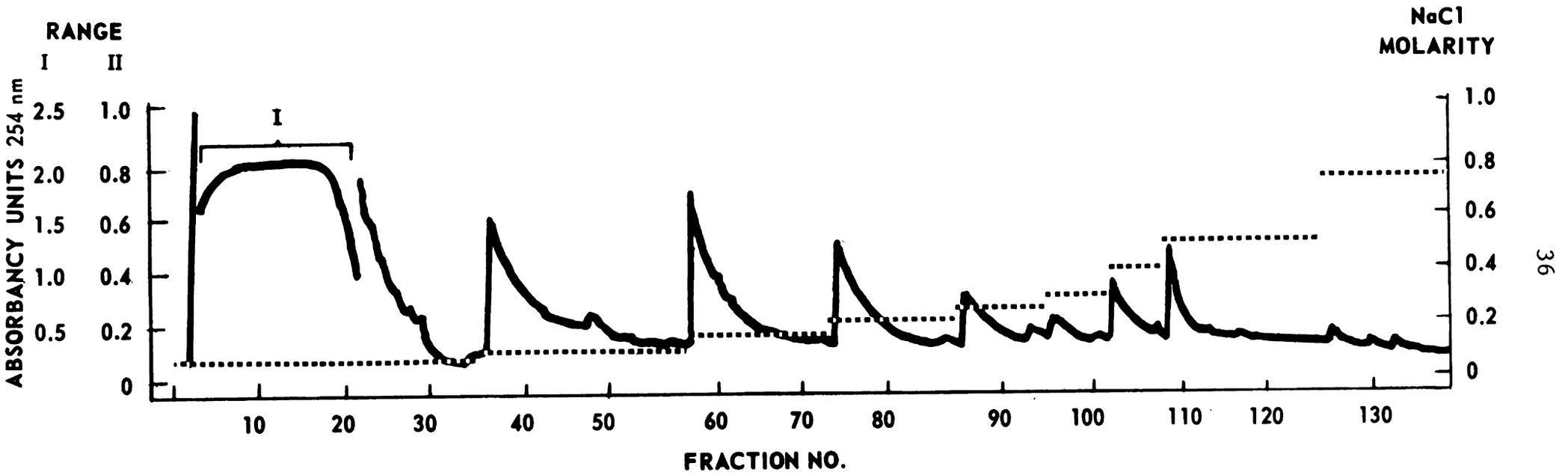

FIGURE 4. Absorbancy at 254 nm of the effluent of concentrated antigens (Figure 5) following aminoethyl cellulose chromatography. The initial absorbancy was measured with range I, 0-2.5 units; the remainder was measured with range II, 0-1.0 units.

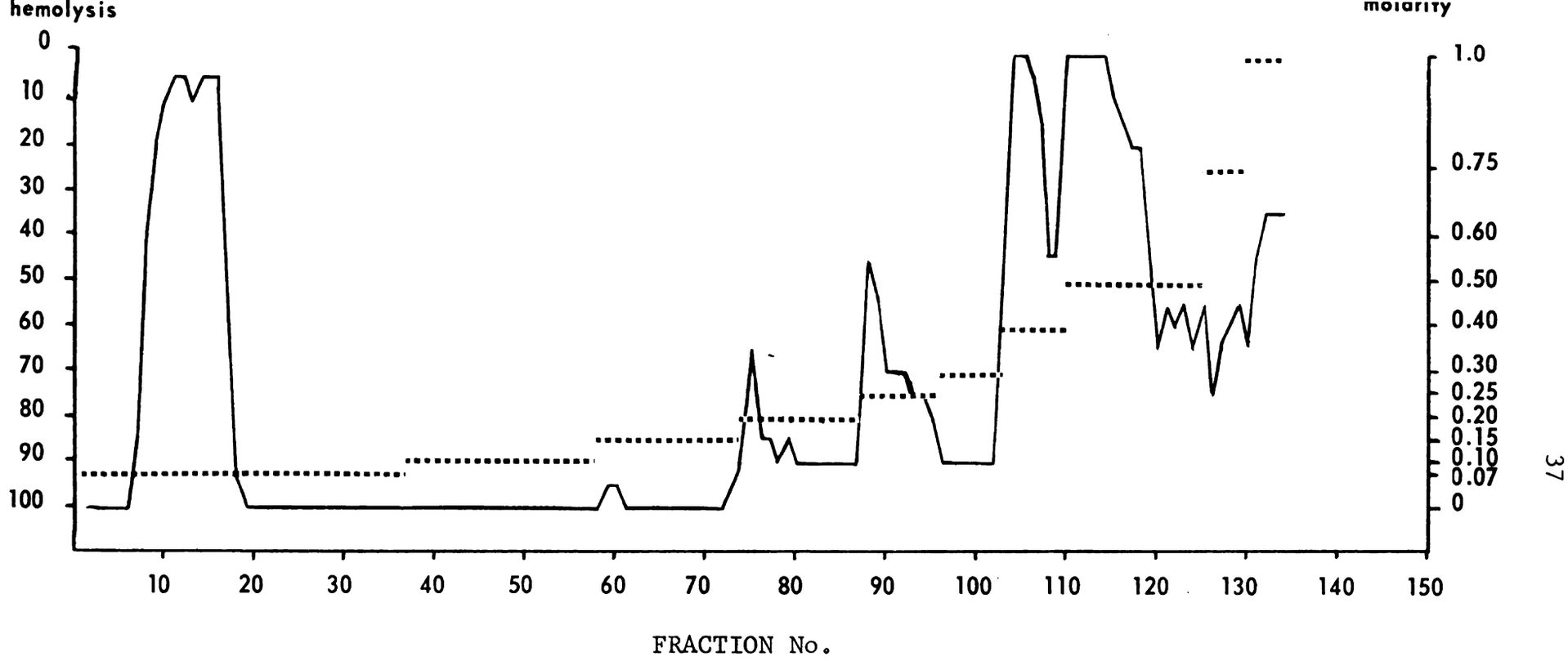

FIGURE 5. Aminoethyl cellulose chromatography of concentrated antigens. Solid line, percent hemolysis; broken line, NaCl molarity.

ECTEOLA and was recovered in 16 ml of effluent, designated soluble (Figure 6). Eight milliliters of effluent having a small amount CF activity were obtained from the 0.1 M NaCl eluate, designated uble "B". The 0.3, 0.4, and 0.5 M NaCl eluates contained low levels CF activity, but in very small volumes.

The pooled 0.2, 0.25, 0.4, and 0.5 M NaCl eluates, "infectious ", from the AE column (Figure 5) had a CF titer of 1, and only as much infectivity as the concentrated antigens which were ied to the column (Table 8). However, the protective potency, ED_{50}, was much higher than was anticipated.

The "infectious pool" volume was 3-fold larger than the volume oncentrated antigens applied to the column. When the assay lts were increased by a factor of 3, the calculated recoveries ; infectivity 1.41%, CF 37.5% and immunogenicity 540%. The calcu- d potency of 675 ED_{50} (3 x 225) clearly indicated the designated ncy of 125 ED_{50} for concentrated antigens applied to the column too low.

It is possible the low infectivity of the "infectious pool" was to the concentration of salt used for elution of virus from the ellulose. Virus suspensions held four days at 4°C in NaCl concen- ions greater than 0.2 M retained less than 40% of their original ctivity (unpublished data).

The two soluble antigens, "A" (nonexchanged) and "B" (0.1 M NaCl te), obtained from the chromatography with ECTEOLA, had measurable ctivities and protective potency EDs_{50} of 19 and 22, respectively le 8).

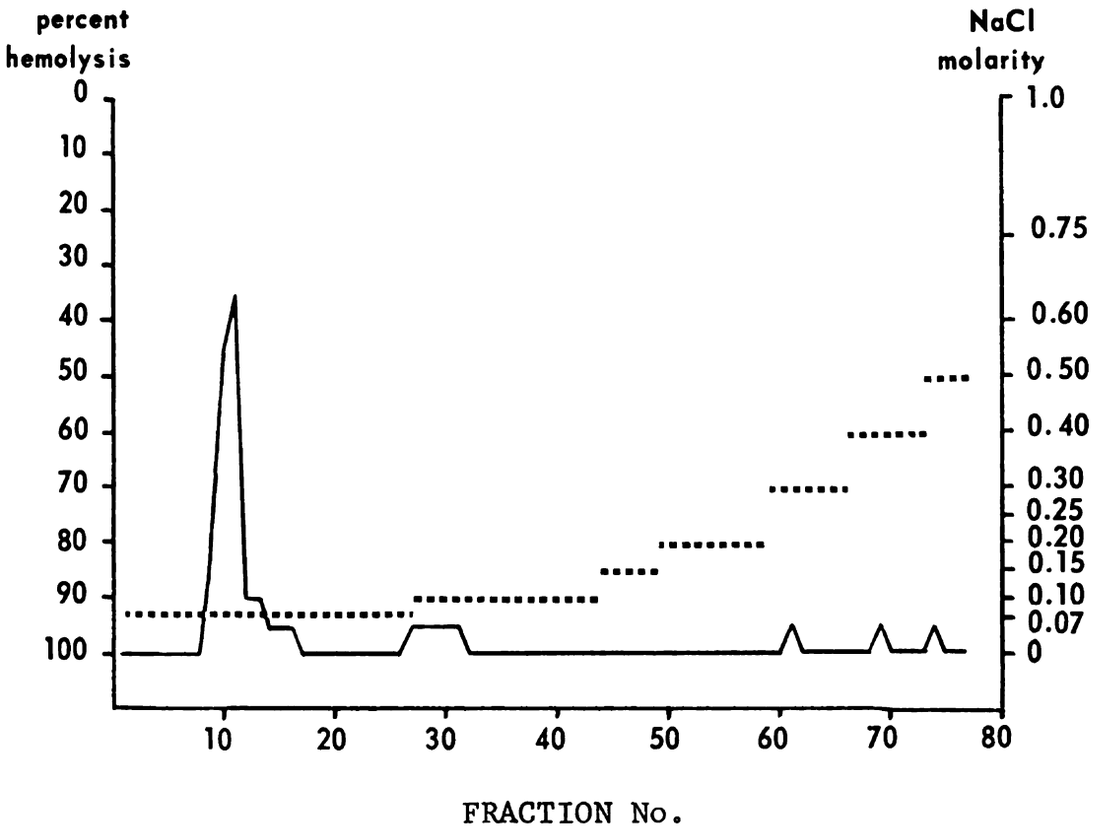

FIGURE 6. ECTEOLA-cellulose chromatography of complement-
ng antigens not previously exchanged by aminoethyl cellulose
concentrated antigens (Figure 5). Solid line, percent
lysis; broken line, NaCl molarity.

TABLE 8. Aminoethyl cellulose and ECTEOLA-cellulose chromatography of concentrated antigens

	CF				Relation to concentrated antigens			
	Percentage of hemolysis[a]	Titer[b]	Infectivity[c]	Protective potency[d]	Volume	CF	Infectivity	Protective potency
Concentrated antigen	0	8	3.83	> 125	1			
Infectious pool[e]	5	1	1.50	225	.33	.125	.0047	< 1.8
Not retained by AE-cellulose	25	1	0	....	.83	.125	0	....
Soluble "A"[f]	40	0	0	19	.94	<.125	0	< .14
Soluble "B"[g]	65	0	0	22	1.88	<.125	0	< .18

[a] Undiluted antigen.

[b] Reciprocal of the highest dilution of antigen producing 30% or less hemolysis.

[c] Log_{10}, LD_{50}/.03 ml.

[d] ED_{50}/ml.

[e] 0.2 M, 0.25 M, 0.4 M, and 0.5 M NaCl eluates from AE cellulose.

[f] Complement-fixing antigens not exchanged by ECTEOLA-cellulose.

[g] Complement-fixing antigens in the 0.1 M NaCl eluate from ECTEOLA-cellulose.

The finding of most of the immunogenicity recovered from the AE-chromatography in the "infectious pool" did not correlate with the finding of 50% of the immunogenicity in the noninfectious 0.05 μm pore diameter filtrate (Table 7).

Chromatography of 0.05 μm pore diameter filtrate. The 0.05 μm pore diameter noninfectious filtrate was chromatographed on AE-cellulose and the chromatogram (Figure 7) compared to that obtained with infectious concentrated antigens (Figure 5). Fifteen milliliters of 0.05 μm pore diameter filtrate (0.14 M Cl^-) were diluted 2-fold, to 0.07 M NaCl, with distilled water and chromatographed with AE-cellulose. Almost the same pattern of nonexchanged CF activity was found (Figure 7). There were four areas of low CF activity; the 0.07 M NaCl wash, and the 0.1, 0.20 and 0.25 M NaCl eluates. The most noticeable difference was the very small amount of CF activity in the 0.4 and 0.5 M NaCl eluates compared to the same eluates from the chromatography of concentrated antigens.

This finding, together with the infectivity of the 0.4 and 0.5 M NaCl eluates from the chromatography of concentrated antigens, indicates infectious virus is retained by AE-cellulose and elutes at relatively high NaCl concentrations. Conversely, noninfectious antigens in the 0.05 μm pore diameter filtrate are not exchanged by AE-cellulose under the conditions used.

Chromatography of dialyzed concentrated antigens. Concentrated antigens had been dialyzed and chromatographed during preliminary work. This work indicated very little additional retention to DEAE occurred at NaCl concentrations lower than 0.07 M.

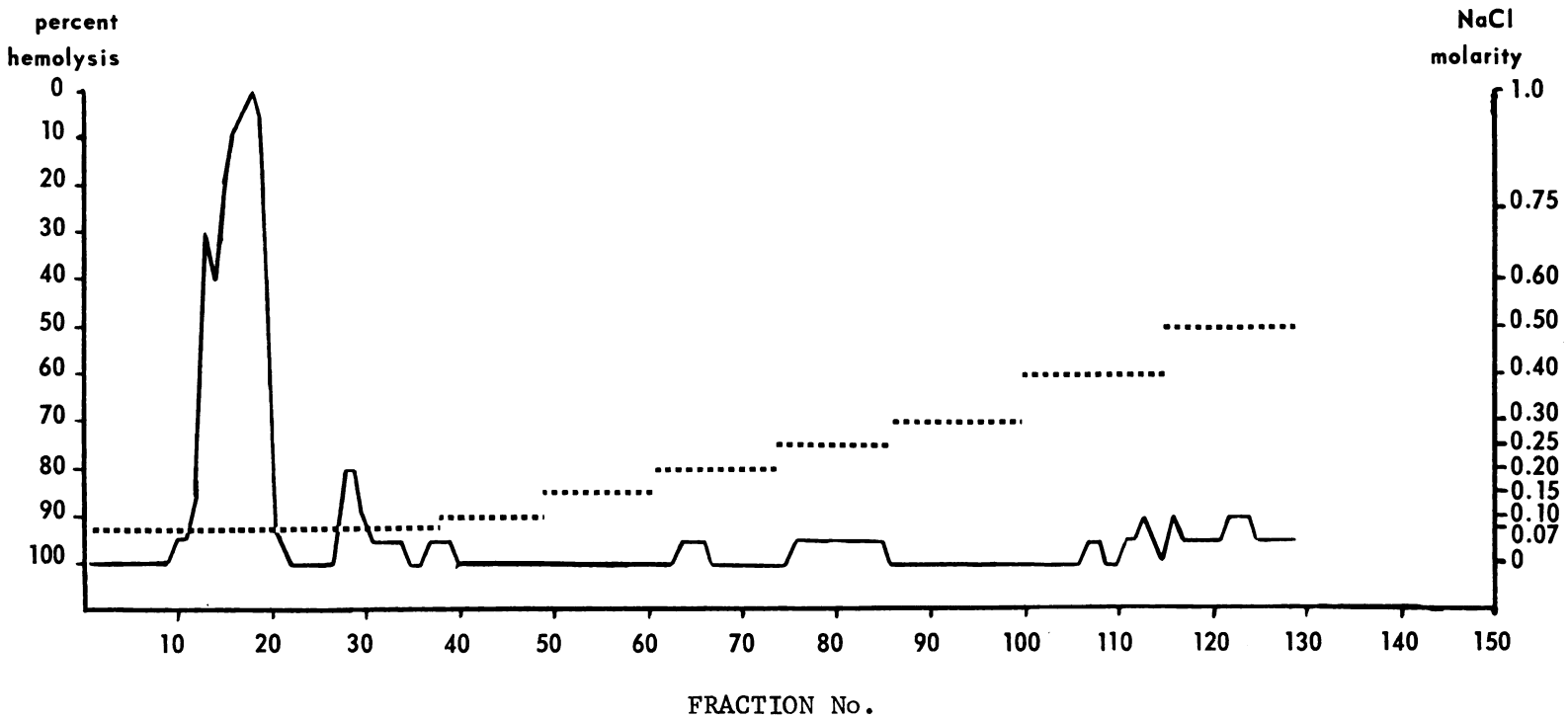

FIGURE 7. Aminoethyl cellulose chromatography of the 0.05 μm pore diameter membrane filtrate of concentrated antigens. Solid line, percent hemolysis; broken line, NaCl molarity.

Exchange by AE-cellulose at NaCl concentrations lower than 0.07 M was examined by extensively dialyzing a sample of concentrated antigens before chromatography. The column was washed with 0.005 M NaCl and eluted with 0.01, 0.05, 0.1 M NaCl, and the remainder of the step wise gradient previously used.

There was a very low CF activity in the nonexchanged effluent (Figure 8). Although values were lower, the remainder of the chromatogram was similar to the chromatogram of concentrated antigens (Figure 5).

The CF titer of concentrated antigens decreased from 8 to 2 during dialysis. This decrease may have been due to adsorption of antigen to the dialysis tubing, although the tubing was washed vigorously at the termination of dialysis. Preferential adsorption to the tubing could have accounted for the low nonexchanged CF activity.

This hypothesis was tested by diluting two milliliters of concentrated antigens 1/10, to lower the NaCl concentration to 0.014, and chromatographing the antigens with AE-cellulose. An area of CF activity was retarded and washed from the column after the major amount of nonexchanged CF material (Figure 9). Additional CF activity was eluted with 0.025 M NaCl. All CF activity was still not exchanged by the cellulose at this low NaCl concentration. However, comparison of the two chromatograms containing reduced Cl^- concentrations verified loss of nonadsorbing CF activity during dialysis (Figures 8 and 9).

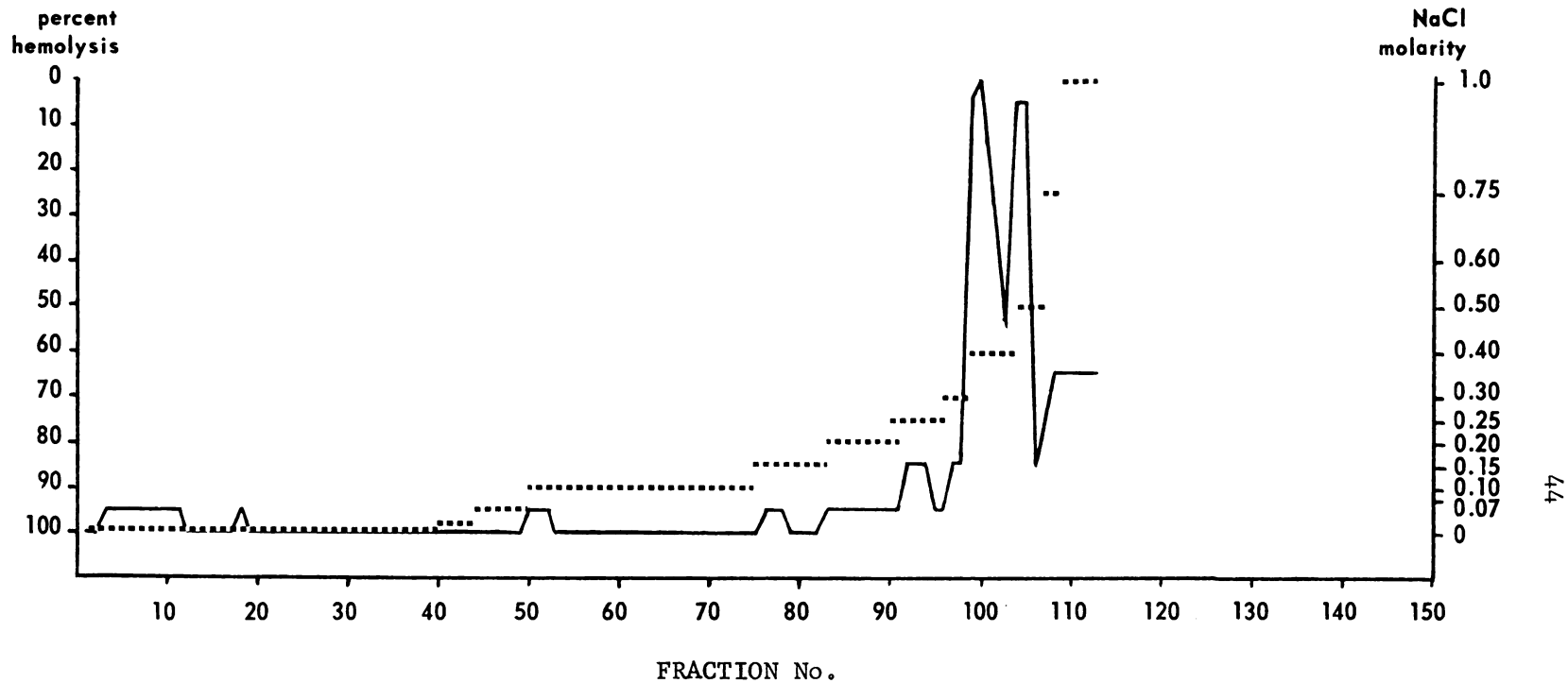

FIGURE 8. Aminoethyl cellulose chromatography of previously dialyzed concentrated antigens. Solid line, percent hemolysis; broken line, NaCl molarity.

Chromatography of concentrated antigens with four additional anion celluloses.

a. ECTEOLA-cellulose. Ten milliliters of concentrated antigens (0.14 M Cl^-) were applied to an ECTEOLA-cellulose column. There was no better exchange of CF material or improved fractionation of the retained CF activity (Figure 10) than was obtained with AE chromatography (Figure 5).

b. PAB- and PEI-cellulose. Complement-fixing antigens were not retained and eluted from these cellulose derivatives.

c. DEAE-cellulose. Ten milliliters of concentrated antigens were diluted 1/10 with distilled water to allow exchange by DEAE-cellulose at a 0.014 M Cl^- concentration. Chromatography differentiated three areas of CF activity during washing of the column (0.014 M NaCl), and seven areas of CF activity during elution (Figure 11).

Chromatography of supernatant fluid and sediment from centrifuged rabies vaccine. Verification of the exchange of rabies virus by AE-cellulose and its elution at higher NaCl concentrations, as well as the nonexchange of soluble, noninfectious CF antigens, was attempted. Inactivated rabies virus which had been sedimented at 125,000 x g for 3 hours and resuspended to 1% of original volume in tissue culture medium containing 0.25% human serum albumin, and supernatant fluids from this centrifugation, were obtained from the viral vaccine production unit of the Michigan Department of Public Health, Bureau of Laboratories.

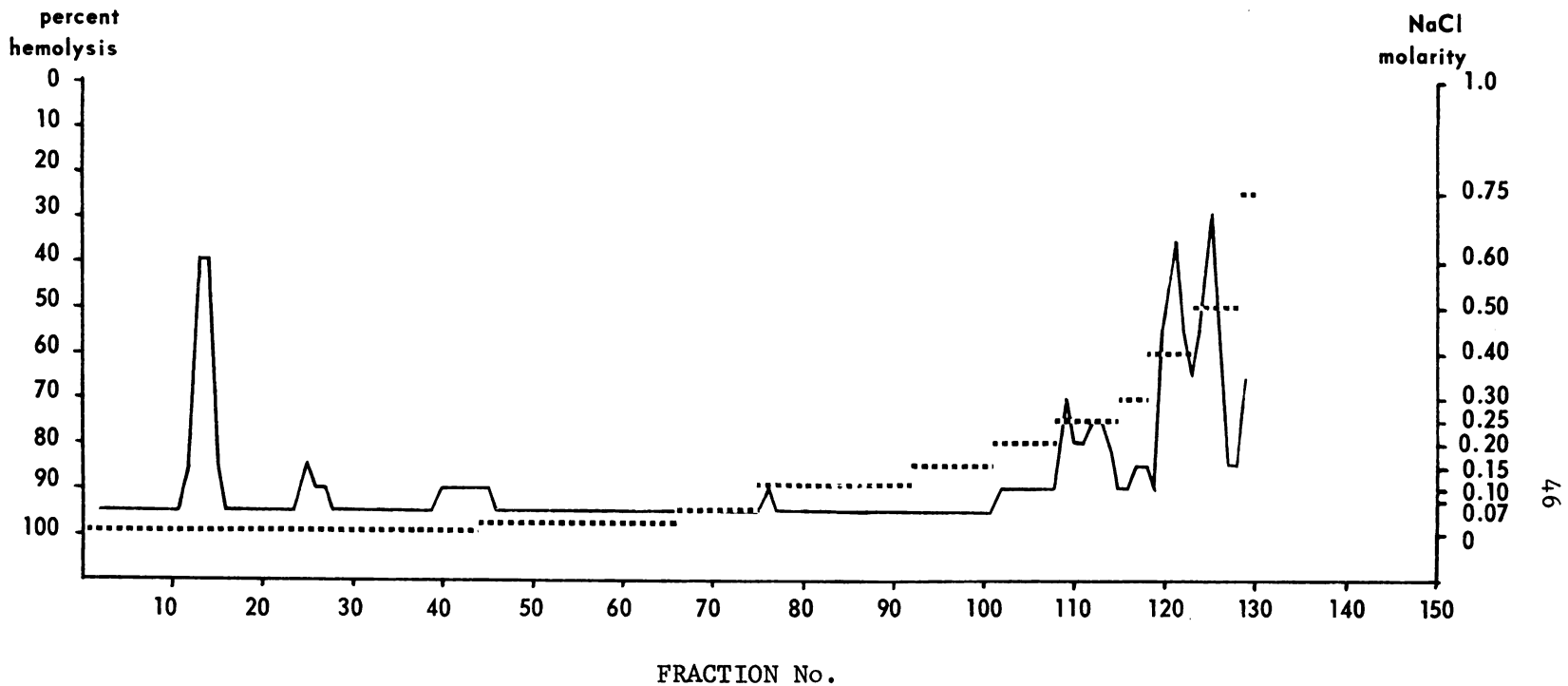

FIGURE 9. Aminoethyl cellulose chromatography of concentrated antigens diluted 10-fold. Solid line, percent hemolysis; broken line, NaCl molarity.

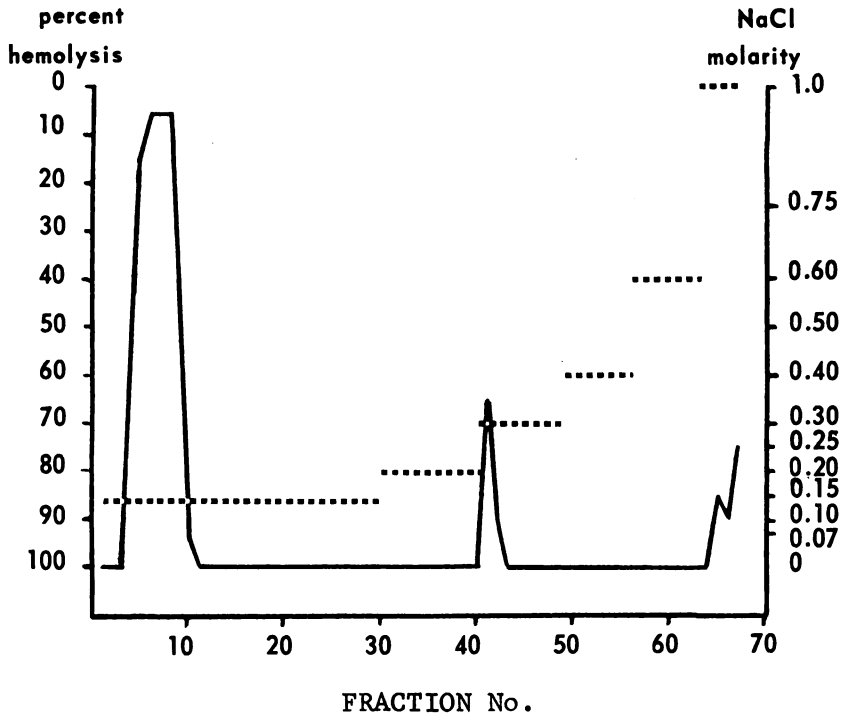

FIGURE 10. ECTEOLA-cellulose chromatography of concentrated antigens. Solid line, percent hemolysis; broken line, NaCl molarity.

Supernatant fluid was concentrated 10-fold by parlodion membrane filtration and passed through a sequence of treated cellulose membranes, terminating with a 0.05 μm pore diameter membrane. This virus-free supernatant fluid, containing historical "soluble antigens", was chromatographed with AE-cellulose. Most of the CF activity was not retained by the ion exchange medium (Figure 12).

Chromatography of virus concentrated by sedimentation was even more dramatic. There was complete exchange of CF activity, and elution with 0.3 M and greater NaCl concentrations (Figure 13). Elution with 0.4, 0.5, 0.6, and 0.75 M NaCl was so great that no fractionation of CF antigens was obtained.

Since DEAE-cellulose chromatography of concentrated antigens had fractionated nonexchanged, noninfectious antigens (Figure 11), concentrated, noninfectious supernatant fluid was chromatographed with DEAE-cellulose. The chromatogram indicated some CF activity was not exchanged, but there was a separation of exchanged material into at least 7 major fractions; the 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, and 0.5 M NaCl eluates (Figure 14).

Chromatography of treated supernatant fluid was also accomplished with ECTEOLA-cellulose. Again, some CF antigen was not retained by the ion exchange medium, however, exchanged antigens were separated into 7 major CF fractions; the 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, and 0.5 M NaCl eluates (Figure 15).

These results indicate the presence of at least 8 soluble, noninfectious antigens, one in the nonexchanged effluent, and seven which were specifically eluted. There were indications that more antigens

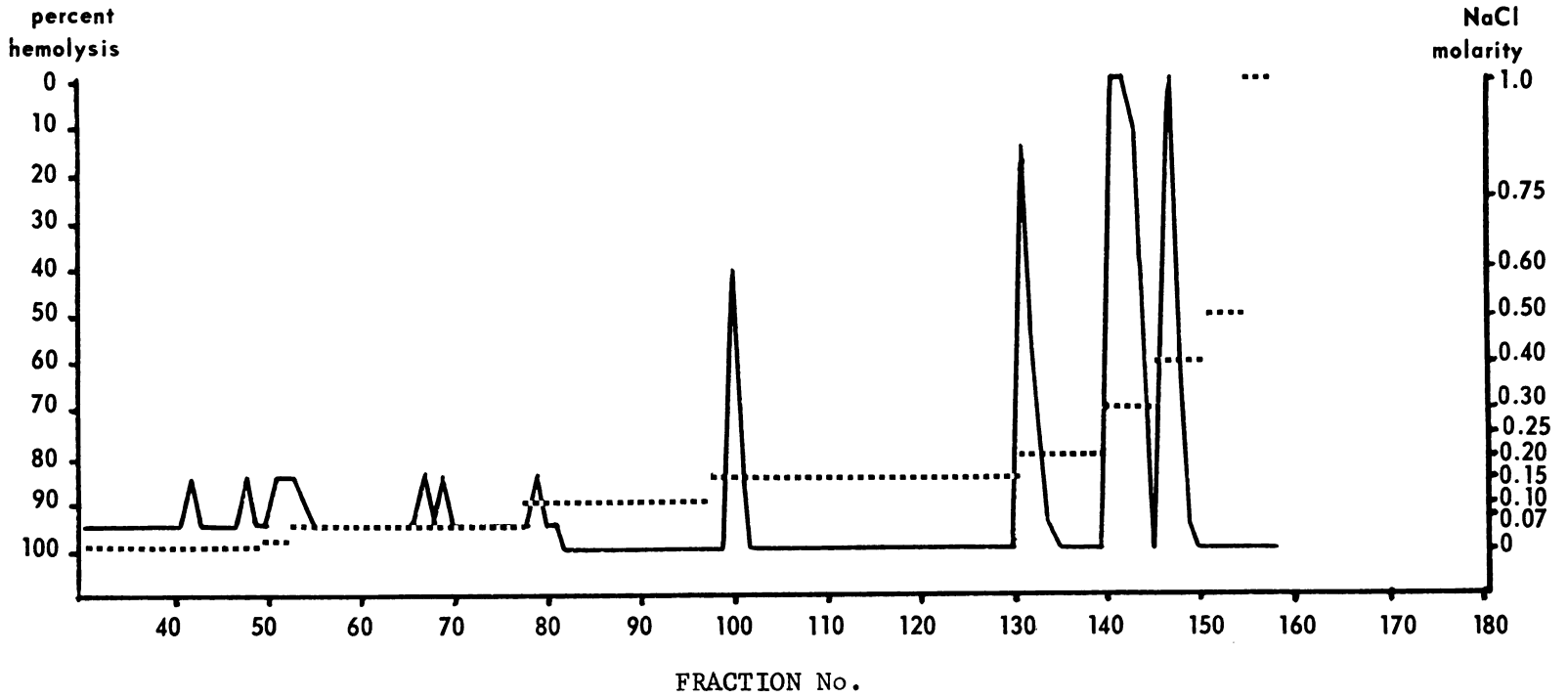

FIGURE 11. Diethylaminoethyl cellulose chromatography of concentrated antigens. Solid line, percent hemolysis; broken line, NaCl molarity.

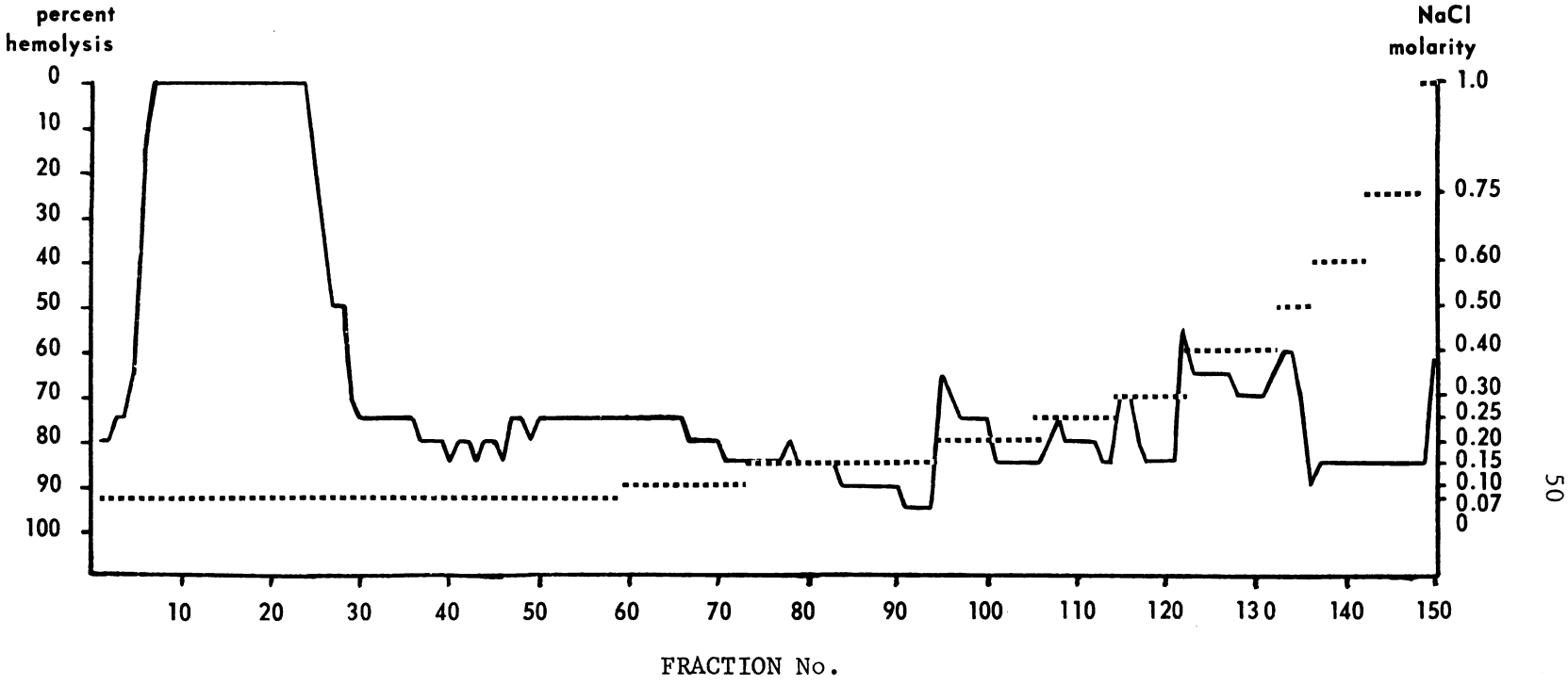

FIGURE 12. Aminoethyl cellulose chromatography of concentrated supernatant fluid following centrifugation of rabies vaccine. Solid line, percent hemolysis; broken line, NaCl molarity.

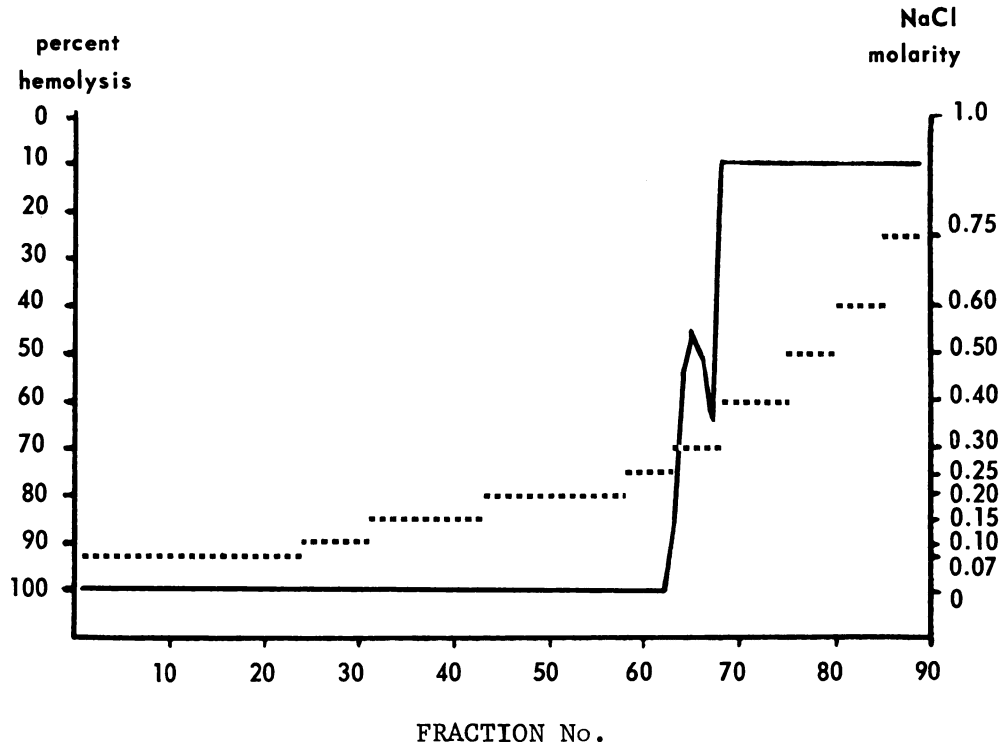

FIGURE 13. Aminoethyl cellulose chromatography of sediment resuspended following centrifugation of rabies vaccine. Solid line, percent hemolysis; broken line, NaCl molarity.

were present, but were not separated. Minor peaks of activity accompanied the major CF activities eluted with 0.1, 0.2, 0.25, and 0.4 M NaCl (Figure 14). One, and possibly two minor peaks of activity accompanied the 0.15 M NaCl eluate, while the 0.4 M NaCl eluate appeared to be two activities eluted at slightly different periods (Figure 15).

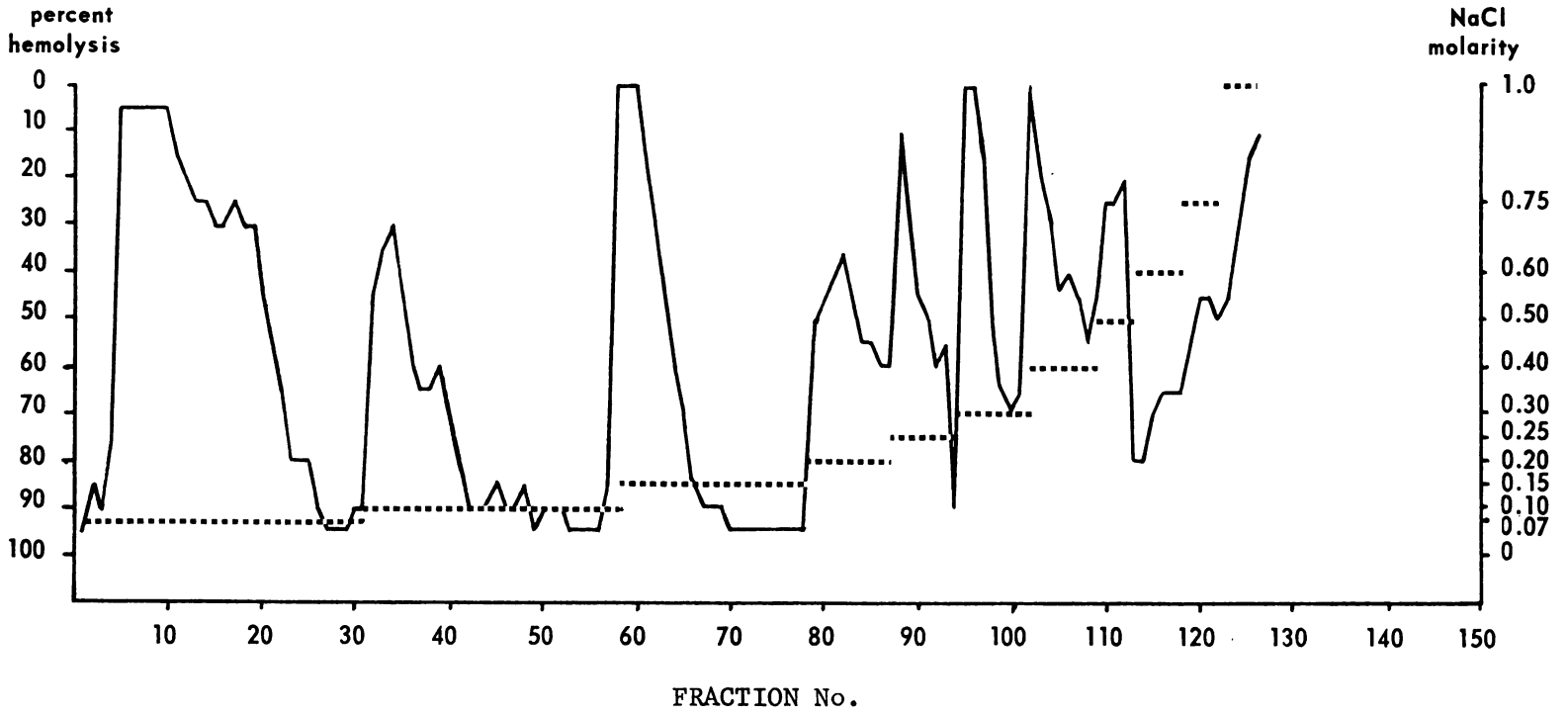

FIGURE 14. Diethylaminoethyl cellulose chromatography of concentrated supernatant fluid following centrifugation of rabies vaccine. Solid line, percent hemolysis; broken line, NaCl molarity.

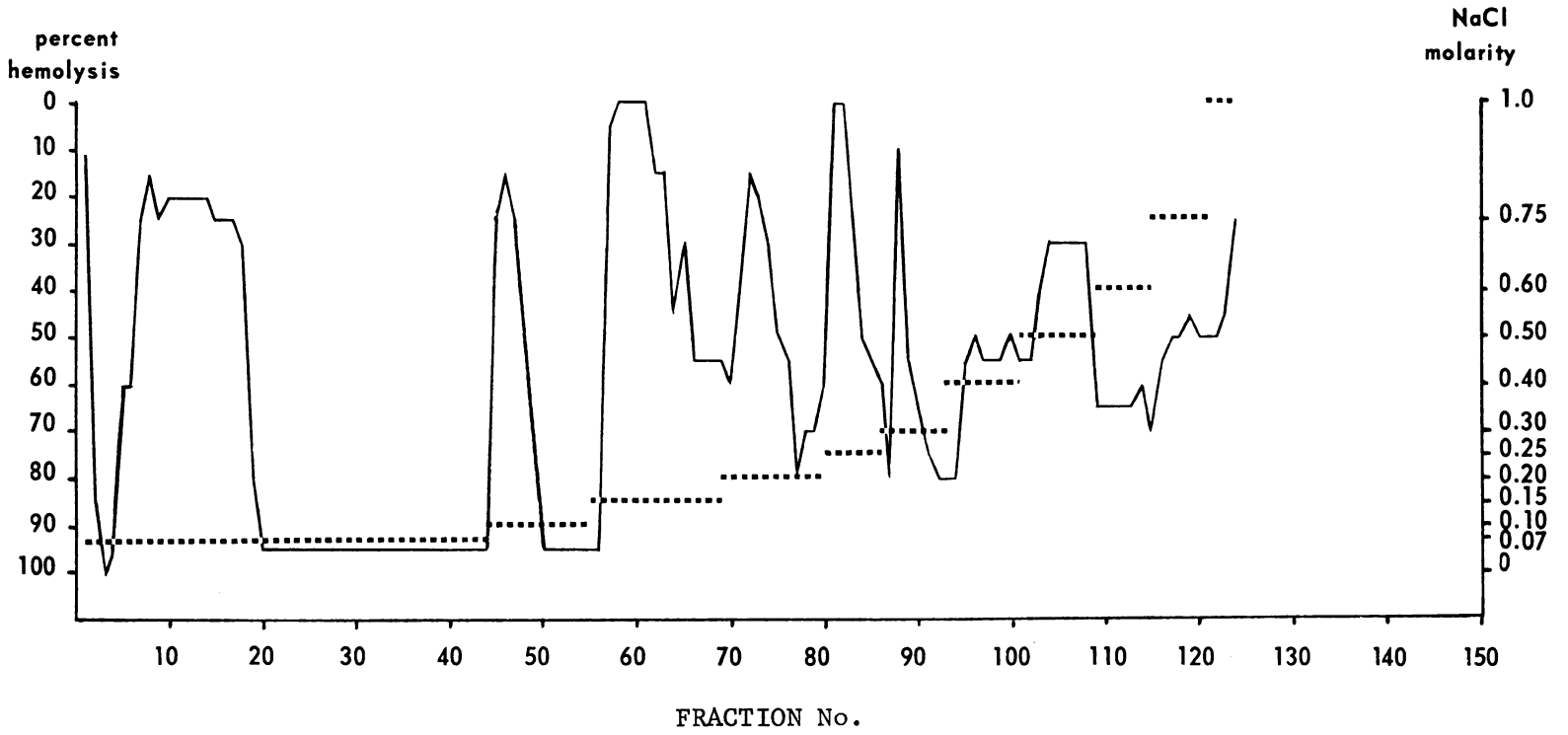

FIGURE 15. ECTEOLA-cellulose chromatography of concentrated supernatant fluid following centrifugation of rabies vaccine. Solid line, percent hemolysis; broken line, NaCl molarity.

DISCUSSION

In PHK cultures infected with rabies virus overt disruption of the cell sheets is not observed and the cells appear to retain their integrity throughout the incubation period. This observation, and the finding that most of the infectivity and immunogenicity is in the culture fluid rather than in the cellular material, indicates there is an orderly release of the antigens through the cell membranes to the fluid medium, without cellular disruption. This implication is supported by observations with electron microscopy of viral particles budding on cell membranes (15, 21).

When it became obvious a concentration of the antigens would be required to enhance CF activity before proceeding with the study, filtration was chosen as a method which was not selective and which probably would concentrate all the various rabies antigens. Almost equivalent concentration of infectivity and CF activity, 89% and 80% respectively and an absence of infectivity and CF activity in the filtrate indicates the concentration was not selective.

Immunogenic assays of supernatant fluids from which most infectious virus had been removed by centrifugation (1, 24) indicated noninfectious CF antigens were immunogenic. However, since these fluids contained small residues of virus, absolute certainty of the protective ability of nonviral CF antigens was lacking. A 0.05 μm

pore diameter filter was used during this study to remove all infectivity from concentrated cultural fluids. The protective ability of the resulting filtrate clearly demonstrates some noninfectious CF antigens are immunogenic.

It is doubtful, though, that 0.05 μm pore diameter filtrates are similar antigenically to supernatants of centrifuged infectious fluids. Usually supernatant fluids contain approximately 10% to 20% of the original protective potency, but, in contrast, the 0.05 μm pore diameter filtrate retained 46% of original potency. One explanation for the greater recovery of immunogenicity following filtration than following centrifugation would be passage through the filter of immunogenic CF antigens which are sedimented from supernatant fluid by centrifugation. It is possible these antigens are fragments of viral coats.

Aminoethyl cellulose chromatography of supernatant fluid and resuspended sediment from rabies vaccine disclosed two interesting correlations: one group of antigens in the vaccine was sedimented during centrifugation, was exchanged by AE-cellulose, and exhibited most of the immunogenicity; the other group of antigens was not easily sedimented by centrifugation, was not exchanged by AE-cellulose, and was not very immunogenic.

With the limited knowledge available, no definitive conclusions can be made concerning the nature of rabies antigens in cultural fluids. However, results of this investigation do provide basis for limited speculation.

There are two major possibilities for the origin of rabies antigens other than virions. They may be excreted along with virus from intact infected cells, or a variety of antigens may be derived from virion breakdown in the culture fluid. No evidence is available to support the first concept. The second hypothesis of virus breakdown in cultural fluid would provide nucleocapsids, nucleocapsid RNA, nucleocapsid protein, viral coats, fragments of coats, and a variety of molecules derived from the viral coats.

This study found that infectious cultural fluids contain immunogenic antigens smaller than virions, but which sediment and chromatograph along with the virus. These results suggest the antigens are viral coat components which originate from the breakdown of virus in the cultural fluids.

SUMMARY

The fluid portion of rabies infected PHK cultures were found to contain the bulk of the infectivity and immunogenicity. These antigenic fluids were concentrated successfully by parlodion membrane filtration. Infectious virus was removed from concentrated fluids by passing them through 0.05 μm pore diameter cellulose membrane filters. The filtrate contained 40% and 45.6%, respectively, of the CF activity and immunogenicity of the original fluid harvests.

Anion exchange cellulose column chromatography separated virus and noninfectious antigens and fractionated the noninfectious antigens. Very little, if any, "soluble", noninfectious CF activity was exchanged by AE-cellulose, but merely passed through the columns. On the other hand, all the infectious, sedimentable CF material was exchanged by the cellulose. Most of the immunogenic antigens contained in infected primary hamster cell culture fluids were recovered in CF material which was adsorbed by and eluted from AE-cellulose.

At least eight CF antigens were separated by ECTEOLA-cellulose and DEAE-cellulose chromatography of "soluble" CF antigens.

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