

AD 714455

Purification, Concentration, and Inactivation of Venezuelan Equine Encephalitis Virus

JACK GRUBER

Medical Sciences Laboratory, Fort Detrick, Frederick, Maryland 21701

Received for publication 8 June 1970

Venezuelan equine encephalitis (VEE) virus was purified and concentrated by chromatography of tissue culture supernatant fluids on diethylaminoethyl-cellulose columns. Stepwise gradient elution studies indicated a broad-elution pattern for the virus, with recovery occurring from 0.05 to 0.7 M NaCl. Optical density, infectivity, hemagglutination (HA), and complement fixation (CF) assays indicated that complete recovery of input virus in highly purified form was possible. Single-step elution with 0.7 M tris(hydroxymethyl)amino:methane-succinate-salt buffer resulted in a virus volume decrease of 85% with a concomitant increase in infectivity and antigenicity. Recoveries consistently equaled or exceeded 100% of the input preparations. Additional purification of column-recovered virus was obtained by sedimentation of pooled virus eluates on 50% sucrose cushions. Exposure of borate saline and 0.5% histidine suspensions of purified VEE virus preparations to 6×10^5 r of gamma radiation resulted in a loss of infectivity for tissue culture and a loss of lethality for weanling and suckling mice. Inactivation was an exponential function of the dosage. In contrast to infectivity, antigenicity (HA and CF) of both saline and histidine preparations was retained after irradiation with doses as high as 6×10^5 r. Purified and irradiated VEE virus preparations have been successfully used for routine serological tests and are being evaluated as vaccines.

The need for adequate protection of laboratory personnel who have contact with Venezuelan equine encephalitis (VEE) virus has long been established. Early attempts to solve this problem resulted in the development of several Formalin-inactivated vaccines (24, 22; Smith et al., *Bacteriol. Proc.*, p. 59, 1954) and, more recently, in one with live attenuated virus (3). Unfortunately, the inactivated VEE vaccines either contained residual infectious virus (29) or were so drastically treated as to confer inadequate protection (3, 14). The live, attenuated vaccine, although conferring good protection in man (14), causes a significant number of vaccinees to display clinical influenza-like symptoms and leukopenia and to manifest transient electrocardiogram abnormalities (1).

In attempts to develop a more effective non-viable VEE vaccine, the applicability of two approaches not previously utilized in conjunction was investigated: purification and concentration of virus by chromatography and inactivation by ionizing radiation. Recent studies indicated that arbovirus preparations of high purity and concentration could be obtained by chromatography on diethylaminoethyl (DEAE)-cellulose columns (16; Fuscaldo et al., *Bacteriol. Proc.*, p. 161, 1969). This procedure seemed appropriate for

obtaining VEE virus preparations with increased specific antigen concentration and minimal non-immunogenic constituents. Other studies have demonstrated that a variety of microbial agents killed by ionizing radiation retain greater antigenicity than do preparations killed by chemicals or heat (6, 7, 20, 33). This method of inactivation also destroys the infectivity of VEE virus while retaining the capacity of the virus to stimulate antibody production (26).

The results of our investigation of purification and concentration of VEE virus and subsequent inactivation by ionizing radiation are presented in this report.

MATERIALS AND METHODS

Virus. The Trinidad strain of VEE virus, originally isolated in guinea pigs from a dorkey brain (23), was used. It had been passaged 13 times in chicken embryos when it was obtained through the courtesy of our colleague William P. Allen. Working seeds for these studies were prepared from a second suckling mouse brain passage as 10% suspensions of infected suckling mouse brains in borate saline.

Infectivity and antigenicity determinations. Assays for virus infectivity were performed by plaque titration in 24-hr chick embryo monolayer tissue cultures and in 10- to 12-g weanling mice. Samples of ir-

radiated virus that failed to produce plaques and were nonlethal for weanling mice following joint intracerebral-intraperitoneal inoculation (0.03 and 0.1 ml) were titrated in suckling mice for residual live virus by a combination intracerebral and intraperitoneal inoculation of 0.02 and 0.03 ml, respectively (26). Fifty per cent lethal dose end points were calculated by the method of Reed and Muench (25). Assays for virus antigenicity were performed by hemagglutination (HA) and complement fixation (CF) tests by using microtiter methods (5, 27). HA was tested for with goose erythrocytes at pH 5.8; the National Communicable Disease Center Laboratory Branch Complement Fixation (LBCF) procedure was employed in complement fixation tests (31).

Tissue culture virus. VEE virus was grown in 24-hr chick embryo monolayer tissue culture, and infected supernatant fluids were harvested by aspiration at 18- to 20-hr postinfection. Tissue culture cellular debris was removed by slow-speed centrifugation and the clarified virus preparations were then dialyzed for 18 hr at 5°C against 10 volumes of water to decrease their salt concentration. This procedure did not materially decrease VEE plaque titer or antigenicity.

DEAE cellulose column chromatography. Columns for chromatography were prepared from 10 g of DEAE-cellulose. Before use they were washed and equilibrated with 0.01 M NaCl in 0.01 M phosphate buffer, pH 7.2. Column dimension ratios, length to diameter, were approximately 2:1. Adsorption of the tissue culture-virus preparation was by free gravity flow, but during development of the column the flow rate was maintained between 5 and 10 ml per min.

Irradiation. Purified virus preparations were irradiated at the National Bureau of Standards through the courtesy of Daniel W. Brown. Suspensions of VEE virus were exposed to gamma radiation from a cobalt-60 source at a dose rate of 7×10^5 r per min. The suspensions were irradiated in 4-ml quantities in polypropylene test tubes (12 by 75 mm). Samples were kept frozen with dry ice during irradiation and were held in the frozen state until tested for infectivity. The different doses of radiation were obtained by varying the time of exposure at the constant dose rate. Control samples of virus were exposed to identical conditions but were not irradiated.

RESULTS

Elution by the stepwise method. Investigation of fractionation of VEE virus was initiated by a study of its elution pattern during a stepwise procedure. After the tissue culture-virus effluent was collected, the virus was eluted with NaCl solutions of increasing concentrations. Fractions of eluates (250 ml each) from each of the various concentrations of NaCl solution were collected. Optical density, infectivity, and HA activity of each fraction were determined (Table 1). Approximately 90% of input virus infectivity and all HA activity were retained by the column before elution was

initiated. In contrast, approximately 80% of the light-absorbing components of the preparation were recovered in the tissue culture effluent. Virus infectivity and antigenicity were not recovered with 0.01 M NaCl elution, whereas additional light-absorbing components were again eluted. VEE virus was eluted in a range of salt concentrations from 0.05 to 0.70 M. The eluates obtained from this range showed different levels of virus content, with the greatest elution occurring at 0.05, 0.10, and 0.5 M concentrations. Table 2, derived from the data in Table 1, shows the per cent recovery of virus infectivity at the various salt concentrations. Essentially all infectivity was recovered by elution through 0.5 M salt concentration. These findings indicated that virus of relatively high purity and concentration might be

TABLE 1. Stepwise elution of Venezuelan equine encephalitis virus from DEAE-cellulose

Material	Volume (ml)	OD ₂₈₀ ^a	Infectivity ^b	HA ^c
Virus prepn....	500	>2.00	8.2	256
Tissue culture-virus effluent....	500	>2.00	6.3	0
0.01 M NaCl....	250	2.00	6.6	0
0.01 M NaCl....	250	0.38	6.0	0
0.05 M NaCl....	250	0.73	8.0	256
0.05 M NaCl....	250	0.31	6.9	8
0.10 M NaCl....	250	0.70	8.2	64
0.10 M NaCl....	250	0.27	6.5	4
0.20 M NaCl....	250	0.60	7.3	8
0.20 M NaCl....	250	0.22	6.5	2
0.50 M NaCl....	250	0.46	7.7	32
0.50 M NaCl....	250	0.09	6.5	2
0.70 M NaCl....	250	0.05	6.7	0
0.70 M NaCl....	250	0.00	6.5	0

^a Optical density at 280 nm.

^b Log₁₀ plaque-forming units/ml on 24-hr chick embryo monolayers.

^c Agglutination of goose erythrocytes at pH 5.8; 0.05 ml.

TABLE 2. Elution of Venezuelan equine encephalitis virus from DEAE-cellulose as a function of salt concentration^a

NaCl concn	Log ₁₀ PFU ^b /ml	Input recovered
M		%
0.01	6.6	1.3
0.05	8.0	33.0
0.10	8.2	49.0
0.20	7.3	5.3
0.50	7.7	14.0

^a Input virus infectivity log₁₀ PFU/ml = 8.2.

^b Plaque-forming units.

obtained by removing contaminating tissue culture material with a salt solution of low molarity and then collecting the virus in one step with a developing buffer of relatively high concentration.

Chromatographic purification of VEE virus. The typical recovery of purified VEE virus by such methodology is shown in Fig. 1. After addition of virus, the columns were washed with 0.01 M NaCl in 0.01 M phosphate buffer, pH 7.2. Then the virus was eluted with a 0.7 M tris(hydroxymethyl)aminomethane-succinate-salt buffer at pH 10. Usually 25-ml fractions were collected. Optical density, infectivity, HA activity, and CF activity of the collected samples were determined. Infectious virus was recovered immediately after the column void volume. Chromatography resulted in a virus volume decrease from 500 to 75 ml (85%), with a concomitant increase in plaque-forming infectivity to represent essentially complete recovery of the input virus. Excellent correlation was found among the various tested properties. Those fractions with high infectivity also had the greatest HA and CF activity. Representative column recoveries of infectivity and serological activity are indicated in Table 3. Recovered infectivity and antigenicity consistently equaled or exceeded 100% of the total input. The mean recovery of infectivity was 115%. The increased infectivity may represent a virus with less aggregation, with greater efficiency for the tissue culture, or may reflect the removal of some infectivity-inhibiting substance.

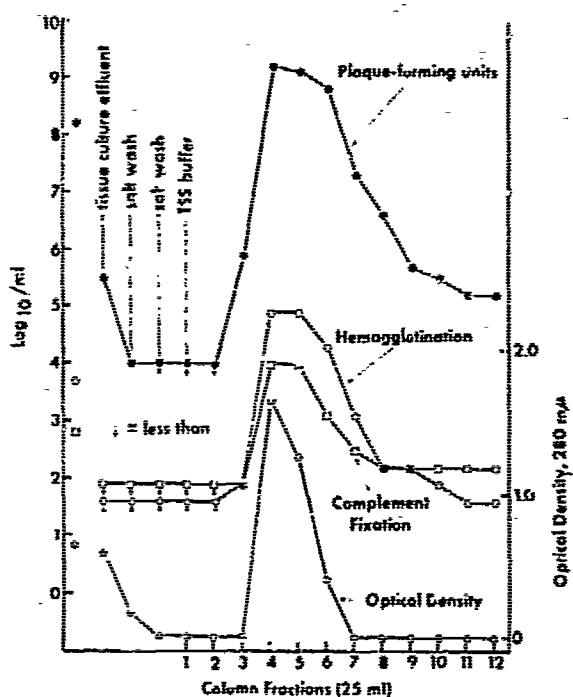


Fig. 1. Recovery of purified Venezuelan equine encephalitis virus by DEAE-cellulose chromatography.

TABLE 3. Recovery of Venezuelan equine encephalitis virus infectivity and serological activity

Expt	Input recovered (%)		
	PFU ^a	HA ^b	CF ^c
A	100	180	170
B	140	180	112
C	106	116	94

^a Plaque-forming units.

^b Agglutination of goose erythrocytes.

^c Complement fixation.

TABLE 4. Spectrophotometric analysis of Venezuelan equine encephalitis virus preparations

Expt	Ratio of optical density at 260 to 280 nm		
	Initial ^a	Final ^b	Increase
A	0.60	0.88	0.29 ^c
B	0.61	0.90	0.29
C	0.72	0.85	0.17
D	0.71	0.89	0.18
			0.23 ^c

^a Dialyzed tissue culture-virus preparations.

^b Recovered DEAE-cellulose column virus pools.

^c Mean ratio increase.

Absorption spectra analysis was also used to monitor VEE virus purification during chromatography. Optical densities at 260 and 280 nm were determined for representative tissue culture-virus preparations and for their recovered column pools. From these values, 260 to 280 nm ratios were determined and are presented in Table 4. In each instance, the 260 to 280 ratio increased after chromatography. The mean ratio increase was 0.23. These increased ratios are assumed to reflect purification of the virus from contaminating tissue culture proteins.

Sedimentation on sucrose cushions. Column-recovered virus was additionally purified by sedimentation of pooled virus eluates on 50% sucrose cushions. Sedimentation was obtained by centrifugation at 27,000 rev/min for 2.5 hr. The thin light-scattering bands recovered from above the sucrose cushions represent highly concentrated and purified virus material. Usually, the final virus preparation was 1/50th the volume of the initial tissue culture material. Typical recoveries are indicated in Table 5. Acceptable recoveries of infectivity and serological activity were obtained by this procedure. The fivefold concentration obtained and the separation from soluble proteins expected from the procedure supported its use.

TABLE 5. Recovery of Venezuelan equine encephalitis virus by centrifugation on a 50% sucrose cushion

Expt	Column pool recovered (%)		
	Infectivity ^a	HA ^b	CF ^c
A	52	20	40
B	119	160	133
C	60	107	58
Mean	77	96	77

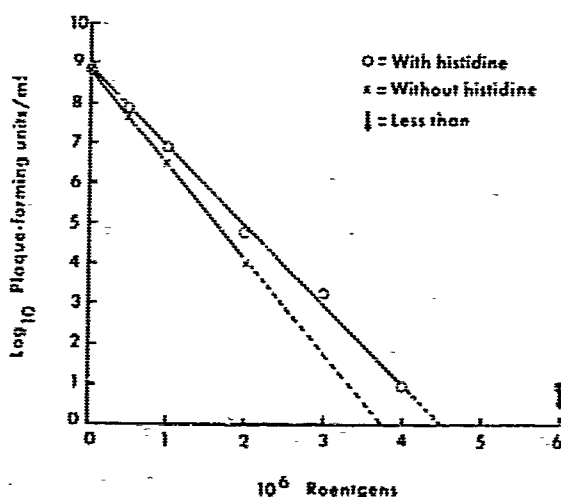
^a Plaque-forming units.^b Agglutination of goose erythrocytes at pH 5.8.^c Complement fixation.

FIG. 2. Inactivation of purified Venezuelan equine encephalitis virus by ionizing radiation.

It should be noted here that 77% mean recovery of column pool infectivity represents essentially, 90% of the total input virus in purified form.

Inactivation. Ring structure compounds inhibit gamma ray inactivation of viruses, and histidine selectivity protects antigenicity more than infectivity (19). The loss of infectivity and antigenicity of purified VEE virus as a function of dose of radiation was studied in borate saline and in 0.5% histidine suspensions. Suspensions were irradiated at a constant dose rate (7×10^4 r per min) for such periods that exposures ranged from 500,000 to 6,000,000 r. The virus preparations were maintained in the frozen state during irradiation to minimize indirect radiation effects. Results of representative infectivity assays in tissue culture are presented in Fig. 2. Inactivation of purified VEE virus was linear, indicating first-order behavior of the reaction. Survival of VEE virus infectivity was enhanced in the presence of histidine. Both saline and histidine preparations lacked plaque-forming capacity after irradiation

at 6,000,000 r. Virus inactivation in histidine was not complete at 4,000,000 r. Virus inactivation was also tested in weanling mice. Typical results obtained and a comparison with a plaque assay are presented in Table 6. Although deaths did not occur in weanling mice inoculated with virus exposed to 4,000,000 and 6,000,000 r, similar inactivation curves indicated potentially live virus in both 4,000,000-r preparations. Intracerebral inoculation of suckling mice confirmed the presence of infectious virus in both saline and histidine preparations irradiated at 4,000,000 r. In contrast, deaths did not occur in newborn mice inoculated with undiluted saline or histidine preparations exposed to 6,000,000 r.

Results of antigenicity assays of saline and histidine suspensions of purified VEE virus are presented in Fig. 3. In contrast to infectivity, the antigenicity of both saline and histidine prepara-

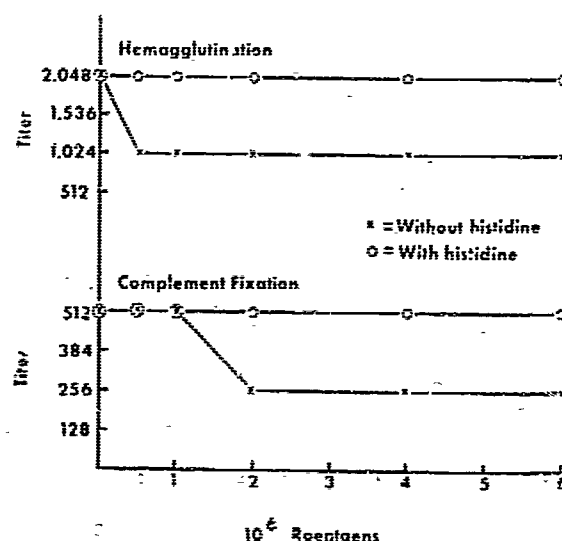


FIG. 3. Retention of antigenicity of purified Venezuelan equine encephalitis virus preparations rendered noninfectious by ionizing radiation.

TABLE 6. Inactivation of purified Venezuelan equine encephalitis virus by ionizing radiation

Radiation dose	Saline prepn		Histidine prepn	
	PFU/ml	MICLD ₅₀ /ml	PFU/ml	MICLD ₅₀ /ml
0 × 10 ⁶	8.9 ^b	10.5	8.9	10.5
0.5 × 10 ⁶	7.7	9.1	7.95	9.2
1.0 × 10 ⁶	6.1	8.0	7.0	8.2
2.0 × 10 ⁶	3.8	5.3	4.95	6.5
4.0 × 10 ⁶	<1.0	<1.5	<1.0	<1.5
6.0 × 10 ⁶	<1.0	<1.5	<1.0	<1.5

^a Plaque-forming units.^b Log₁₀ values.

tions of purified VEE virus was retained after irradiation at doses as high as 6,000,000 r. Histidine suspensions of purified VEE virus completely retained their capacity to produce hemagglutination and complement fixation. At 6,000,000 r, the dose at which all assay systems indicated complete loss of infectivity, titers were identical to those of nonirradiated control preparations. Saline suspensions were similarly active. Although frequently an initial decrease in titer occurred after exposure to low doses of gamma radiation (as indicated in the figure), inactivation did not continue linearly, and a high level of antigenicity was maintained throughout exposure to the higher doses. In these instances, at 6,000,000 r, 50% of the HA and CF properties were retained. More commonly, the saline-virus preparations behaved like those of histidine and no decrease in titer occurred at doses as high as 6,000,000 r.

In vitro serology. Irradiated VEE antigens exposed to 6,000,000 r were employed in our laboratory in CF tests of several animal sera. Results were similar to those obtained with live virus preparations and with saline extracts of infected suckling mouse brains inactivated with 0.3% beta-propiolactone. No change in CF or HA titer was noted with column-purified antigens stored for 3 weeks at 5°C, and only a fourfold decrease in titer was noted after 10 weeks. Studies are currently in progress to evaluate the immunogenicity of these purified VEE virus preparations.

DISCUSSION

Since the initial description in 1958 of the use of ion-exchange adsorbents to purify and concentrate animal viruses (9), a wide variety of viruses, viral components, and viral antigens have been purified by DEAE cellulose chromatography (4, 8, 12, 13, 15, 17, 32). With the arboviruses, however, most investigators using chromatography for purification have employed calcium phosphate (2, 28, 30), and DEAE cellulose has been only rarely used. Nicoli (16) investigated the isolation of soluble hemagglutinins of several arboviruses by DEAE-cellulose chromatography and reported the elution of group A Sindbis virus hemagglutinin at a salt concentration range of 0.3 to 0.5 M. Seventy-one per cent of the hemagglutinin fixed on the column was recovered, and the serological activity was coincident with infectivity. Fuscaldo et al. (Bacteriol. Proc., p. 161, 1969) reported the purification of tissue culture-grown Eastern equine encephalitis (EEE) virus by single-step DEAE-cellulose chromatography and subsequent sucrose cushion and gradient banding. They reported that more than 90% of input virus infectivity was recovered. In the present work, gradient elution of tissue culture-grown VEE

virus occurred at salt concentrations similar, although not identical, to that for Sindbis virus, and single-step elution and sucrose cushion banding resulted in recovery of infectious virus equal to that reported for EEE virus. These results suggest that DEAE-cellulose chromatographic purification is applicable to a variety of arboviruses and that the procedure deserves more attention and wider use.

No naturally occurring soluble hemagglutinins have been reported for group A arboviruses. This investigation substantiates that premise for VEE virus. The recovery of HA activity during chromatography was always coincidental with infectivity. No serological activity was found in tissue culture effluents or in fractions free from infectivity. A direct relationship between the level of HA activity and virus infectivity was clearly demonstrated. Figure 1 illustrates the rise and fall of HA titer coincident with virus infectivity, and gradient elution data (Table 1) further substantiate this relationship over a range of salt concentrations. High, moderate, and low levels of HA were always obtained in eluants of high, moderate, or low levels of infectivity.

The elution of infectious VEE virus at several different salt concentrations during gradient chromatography indicates an apparently heterogeneous binding between the virus population and DEAE cellulose. Such heterogeneity may be a property of the adsorbent employed. Heterogeneous binding had not been reported for VEE virus but such a characteristic had been noted for elution of adenovirus type 2-infected tissue culture fluids from DEAE cellulose (17). In that study, 15, 20, and 15 to 20% of the infectious virus was recovered by gradient elution at 0.5, 0.75, and 1.0 M salt concentrations, respectively. However, in contrast to this present work, the quantitative recovery of adenovirus was not obtained.

Two parameters were used in this study to monitor VEE virus purification by chromatography. With the first, optical density measurements at 280 nm during gradient elution verified the removal of substantial tissue culture material during adsorption and salt washing of the column before the specific elution of virus. Secondly, absorption spectra analysis at 260 and 280 nm of chromatographed preparations provided evidence suggesting purification of viral nucleic acid elements from contaminating proteins. The subsequent sedimentation of column-purified virus on sucrose cushions provides the final preparation with an additional degree of freedom from tissue culture contaminants.

Irradiation of VEE virus under conditions minimizing indirect radiation effects produced linear inactivation of virus infectivity. These re-

sults are consistent with those reported previously for VEE virus (26) and for a variety of other viruses after gamma radiation, including vaccinia, St. Louis encephalitis, Western equine encephalitis (WEE), poliovirus (10), and rubella virus (11). These data are also consistent with results obtained with several viruses irradiated in the fluid state, including influenza A and B, mumps, herpes simplex, and WEE (18, 21). The level of radiation required to completely inactivate purified VEE virus is less than that previously reported for another VEE virus preparation (26). However, this is in keeping with results of earlier studies demonstrating that several purified viruses were inactivated at lower doses than their respective crude preparations (10). The reduction in the inactivation rate of infectious VEE virus provided by histidine agrees with results of prior studies with influenza A virus (19). However, the potential protective effect of histidine on *in vitro* antigenicity was not determined, since the saline preparation itself frequently retained complete activity even after exposure to 6,000,000 r.

ACKNOWLEDGMENT

The valuable technical assistance of Duane Birrell is acknowledged.

LITERATURE CITED

- Alevisatos, A. C., R. W. McKinney, and R. D. Feigin. 1967. Live, attenuated Venezuelan equine encephalomyelitis virus vaccine. I. Clinical effects in man. *Amer. J. Trop. Med. Hyg.* 16:762-768.
- Ardoin, P., and D. H. Clarke. 1967. The use of sonication and of calcium-phosphate chromatography for preparation of group C arbovirus hemagglutinins. *Amer. J. Trop. Med. Hyg.* 16:357-363.
- Berge, T. O., I. S. Banks, and W. D. Tigrett. 1961. Attenuation of Venezuelan equine encephalomyelitis virus by *in vitro* cultivation in guinea-pig heart cells. *Amer. J. Hyg.* 73:209-218.
- Brown, F., and B. Cartwright. 1959. Fractionation of the virus of foot-and-mouth disease by chromatography on DEAE-cellulose. *Biochim. Biophys. Acta* 33:343-346.
- Clarke, D. H., and J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Amer. J. Trop. Med. Hyg.* 7:561-573.
- Donaldson, D. M., and J. R. Mitchell. 1959. Immunization against Ehrlich's ascites carcinoma with X-irradiated tumor cells. *Proc. Soc. Exp. Biol. Med.* 101:204-207.
- Gordon, M., D. M. Donaldson, and G. G. Wright. 1964. Immunization of mice with irradiated *Pasteurella tularensis*. *J. Infect. Dis.* 114:432-440.
- Haruna, I., H. Yagi, R. Kono, and I. Watanabe. 1961. Separation of adenovirus by chromatography on DEAE-cellulose. *Virology* 15:264-267.
- Hoyer, B. H., E. T. Bolton, R. A. Ormsbee, G. LeBouvier, D. B. Riner, and C. L. Larson. 1958. Mammalian viruses and rickettsiae. *Science* 127:859-863.
- Jordan, R. T., and L. L. Kempe. 1956. Inactivation of some animal viruses with gamma radiation from cobalt-60. *Proc. Soc. Exp. Biol. Med.* 91:212-215.
- Kenny, M. T., K. L. Albright, J. B. Emery, and J. L. Bittle. 1969. Inactivation of rubella virus by gamma radiation. *J. Virol.* 4:807-810.
- Klenpeter, H. G., and H. G. Pereira. 1959. Study of adenovirus antigens fractionated by chromatography on DEAE-cellulose. *Virology* 9:536-543.
- McCrea, J. F., and J. O'Loughlin. 1959. Separation of vaccinia hemagglutinin from infectious virus particles by chromatography on DEAE column. *Virology* 8:127-129.
- McKinney, R. W., T. O. Berge, W. D. Sawyer, W. D. Tigrett, and D. Crozier. 1963. Use of an attenuated strain of Venezuelan equine encephalomyelitis virus for immunization in man. *Amer. J. Trop. Med. Hyg.* 12:597-603.
- Nakajima, H., S. Tanaka, and C. Ushimi. 1968. Fractionation of equine infectious anemia virus by diethylaminoethyl cellulose chromatography and sucrose density gradient centrifugation. *Nat. Inst. Anim. Health Quart.* 8:57-63.
- Nicoli, J. 1965. Chromatographie sur celluloses échangeuses d'ions et gel-filtration sur agarose d'arbovirus et de produits extraits de ces arbovirus. *Ann. Inst. Pasteur* 105:695-710.
- Phillipson, L. 1960. Separation on DEAE cellulose of components associated with adenovirus reproduction. *Virology* 10:459-465.
- Polley, J. R. 1961. Preparation of non-infective soluble antigens with gamma radiation. *Can. J. Microbiol.* 7:135-139.
- Polley, J. R. 1961. Factors influencing inactivation of infectivity and hemagglutinin of influenza virus by gamma radiation. *Can. J. Microbiol.* 7:535-541.
- Polley, J. R. 1962. The use of gamma radiation for the preparation of virus vaccines. *Can. J. Microbiol.* 8:455-459.
- Polley, J. R., and M. M. Guerin. 1968. Preparation of a stable noninfective western equine encephalomyelitis virus antigen from allantoic fluid. *Can. J. Microbiol.* 14:1017-1020.
- Randall, R., F. D. Maurer, and J. E. Smadel. 1949. Immunization of laboratory workers with purified Venezuelan equine encephalomyelitis vaccine. *J. Immunol.* 63:313-318.
- Randall, R., and J. W. Mills. 1944. Fatal encephalitis in man due to the Venezuelan virus of equine encephalomyelitis in Trinidad. *Science* 99:225-226.
- Randall, R., J. W. Mills, and L. L. Engel. 1947. The preparation and properties of a purified equine encephalomyelitis vaccine. *J. Immunol.* 55:41-52.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* 27:493-497.
- Reitman, M., and H. R. Tribble, Jr. 1967. Inactivation of Venezuelan equine encephalomyelitis virus by γ -radiation. *Appl. Microbiol.* 15:1456-1459.
- Sewer, J. L. 1962. Application of a microtechnique to viral serological investigations. *J. Immunol.* 89:320-321.
- Smith, C. E. G., and B. Holt. 1961. Chromatography of arthropod-borne viruses on calcium phosphate columns. *Bull. W. H. O.* 24:749-759.
- Smith, D. G., H. K. Mantay, R. G. Marshall, and J. C. Wagner. 1956. Venezuelan equine encephalomyelitis. Laboratory aspects of fourteen human cases following vaccination and attempts to isolate the virus from the vaccine. *Amer. J. Hyg.* 63:150-164.
- Taverner, J., J. H. Marshall, and F. Fulton. 1958. The purification and concentration of viruses and virus soluble antigens on calcium phosphate. *J. Gen. Microbiol.* 13:451-461.
- U.S. Public Health Service. 1965. Standardized diagnostic complement fixation method and adaptation to micro test. Public Health Monograph 74. U.S. Government Printing Office, Washington, D.C.
- Wilcox, W. C., and H. S. Ginsberg. 1961. Purification and immunological characterization of types 4 and 5 adenovirus-soluble antigens. *Proc. Nat. Acad. Sci. U.S.A.* 47:512-526.
- Wright, G. G., N. S. Swack, and J. R. Mitzel. 1969. Antigenicity of psittacosis vaccines killed by ionizing radiation. *Proc. Soc. Exp. Biol. Med.* 131:858-860.