Concentration of Dilute Virus Suspensions by Alum Flocculation. (22607)

ROBERT E. STEVENSON, S. L. CHANG, N. A. CLARKE, AND P. W. KABLER.
(Introduced by Isaac Ruchman.)
Robert A. Taft Sanitary Engineering Center, Public Health Service, Cincinnati, Ohio.

Most chemical and physical methods for virus concentration and/or purification have been developed for treatment of potent rather than weak virus suspensions. Melnick's(1) procedures for detection of poliomyelitis and Coxsackie viruses in sewage are expensive and complicated. In this laboratory, treatment by Kelly's(2) ion-exchange resin absorption technic of waters lightly contaminated with Coxsackie virus failed to yield virus.

A better solution to the problem of concentrating dilute virus suspensions was suggested by an observation that the floc complex formed in the aluminum sulfate flocculation of artificially contaminated water contained in excess of 99% of added Coxsackie virus and that significant amounts of virus could be recovered from the floc. This observation confirmed the findings of Chang, *et al.*(3), who recovered bacterial virus from the floc complex.

Aluminum hydroxide, either as a preformed gel or as a coprecipitate, has been used for adsorption of various toxins, bacteria and viruses. Schaeffer and Brebner(4) and Sabin (5) used aluminum hydroxide gel for adsorption of poliomyelitis virus from tissue suspensions and they determined pH optima for adsorption and elution. Although no quantitative results were reported in their papers, our experience with preformed floc or aluminum hydroxide gel indicates that recovery by this method is not high.

This communication reports a sensitive and effective, yet convenient and inexpensive method for concentration of Coxsackie virus from dilute suspensions.

Materials and methods. Virus. Group A, type 2 Coxsackie virus of the 16th or 17th mouse passage was used as the source of virus. A 20% suspension of infected muscle, centrifuged at 2,000 rpm for 30 minutes to remove large tissue particles, served as stock virus and was diluted in 1% peptone (Difco) for use. The stocks titered 1 X 10^6LD_{50}/0.02 ml and 0.89 X 10^6LD_{50}/0.02 ml. These values were determined from titrations with at least 200 mice for each assay.

To serve as an additional check on virus titers, a dilution tube containing an estimated 1.0 or 0.5 LD_{50} was used for animal inoculation and for preparation of successive dilutions. Thus, each series of dilutions employed in seeding the experimental mixtures was represented by a measurable control. Since the theoretical dosage in the initial mixture before flocculation could not be measured except by inoculation of 100 or more mice, yields of virus were then compared to the measurable control titer in order to estimate percentage recovery.

Reagents and Glassware Cleaning Procedures. 1. All glassware which came into contact with virus was soaked overnight in a solution of Hemo-Sol (Meinicke Co., N.Y.) in order to remove its virus adsorbing capacity [Chang(3)], rinsed 5 times in cold tap water, and once in distilled water before sterilization. 2. The initial pH of the flocculation mixtures (pH 6.2-6.5) was controlled by the ratio of $NaHCO_3$, to $Al_2(SO_4)_3 \cdot 18H_2O$ and was adjusted by means of pH determinations on virus-free controls. 3. Reagents: $NaHCO_3$, 0.1 M; $Al_2(SO_4)_3 \cdot 18H_2O$, 1% solution; Tryptose Broth (Difco), 2% solution; SiO_2 (Activated Silica, 200 mesh, Davison Chemical Corp.), 1% suspension. Reagents were sterilized by autoclaving at 15 lb for 15 minutes except solutions containing sodium bicarbonate. The latter were sterilized by filtration through an 0-3 Selas filter. $NaHCO_3$, 0.1 M; Na_2CO_3, 0.1 M; and HCl, 0.1 N were used to formulate the buffers used for recovery of virus from the floc. The pH values of the solutions were checked with a glass electrode pH meter both before and after filtration.

Flocculation procedures. Into a 600 ml

beaker, 370.8 ml of sterile distilled water, 0.4 ml of SiO_2 suspension, 10 ml of tryptose broth, 10 ml of $NaHCO_3$, and 1.0 ml of diluted virus were added in order. A sterile glass paddle-type stirrer was then placed inside the beaker, the assembly connected to a stirring apparatus and after stirring at 81 rpm for 2 minutes, 7.8 ml of $Al_2(SO_4)_3 \cdot 18H_2O$ (7.8 ml for 100 parts per million) was added, and stirring resumed. Floc formation started within one or 2 minutes and continued with progressive increase in particle size. After 30 minutes of stirring, the stirring rod was removed from the beaker and the floc allowed to settle for 60 to 90 minutes. Speed of stirring proved to be critical. Higher speeds tended to break up floc particles and made settling very slow. After settling occurred, the supernatant fluid was drawn off by means of suction, care being taken not to disturb the floc. The floc was then transferred to a 10 ml mixing cylinder* adapted to a 100 ml centrifuge shield and centrifuged at 1,500 rpm for 5 minutes, to pack the floc. After the removal of the supernatant fluid, the floc was resuspended in a buffer of appropriate pH to a volume of 10 ml.

After standing 1 to 3 hours at room temperature with intermittent agitation, the floc was again centrifuged at 1,500 rpm for 5 minutes and the supernate collected and used for animal inoculation.† One to 3-day-old suckling mice were inoculated subcutaneously with sample material in 0.02 ml quantities and were observed for signs of Coxsackie virus infection for at least 10 days. Methods employing ion-exchange resin were those described by Kelly(2) and were included for a comparison of the two methods.

Results. Using the same virus inoculum, the ion-exchange method was compared to the flocculation method, using 60, 80, and 100 ppm and one and 3 hour redispersing times in the latter. The flocculation method resulted in higher recoveries of virus in all combinations tested (Table I).

TABLE I. Comparison of Alum Flocculation and Ion-Exchange Resin Concentration.

Temp., 26-28°C; pH of water after flocculation, 6.3; pH of buffer used for separation, 8.0; estimated initial virus dosage, .025 LD_{50}/.02 ml; estimated final virus dosage, 1.0 LD_{50}/.02 ml (40 × conc.).

Exp.	Conc. of aluminum sulfate, ppm	Floc redispersing time, hr	Mice, infected /total	% mortality
1	60	1	21/43	48.8
		3	13/38	34.2
	80	1	26/54	48.1
		3	8/22	36.3
	100	1	28/53	52.8
		3	13/28	46.4
	Control at estimated 1 LD_{50} per .02 ml		13/20	65.0
2	60	1	5/14	35.7
		3	3/17	17.6
	80	1	7/40	17.5
		3	11/40	27.5
	100	1	7/33	21.2
		3	17/45	37.7
	Ion-exchange-resin method (40× conc.)		2/33	6.1
	Control at estimated 1 LD_{50} per .02 ml		20/46	43.5

The flocculation results in Table I were examined by a series of statistical significance tests. Since Exp. 2 showed lower percent mortality than Exp. 1, the data for these 2 tests were handled separately, except in one case described below.

In all cases the following chi-square (χ^2) test was applied:

$$\chi^2 = \frac{\Sigma \frac{x_i^2}{n_i} - \frac{(\Sigma x_i)^2}{\Sigma n_i}}{\hat{p}\,(1-\hat{p})}$$

with degrees of freedom equal to one less than the number of percentages compared, where x_i denotes the number of mice killed out of n_i inoculated, and $\hat{p} = \Sigma x_i / \Sigma n_i$ denotes the average proportion response over the conditions being compared. [See Cochran(6)]. Responses were compared under each of the

* Kontes Glass Co., Vineland, N. J.

† Volume relationships in the procedure outlined resulted in a 40 fold concentration and were selected for convenience. Other volumes may be selected so that concentrations of 100 fold or greater may be obtained. For practical use, however, concentrations of greater than 200 fold are difficult because of the floc volume and the molarity of the buffer.

TABLE II. Effect of pH of Floc-Redispersing Fluid on Recovery of Coxsackie Virus from Water Concentrated 40 Times by Alum Flocculation Method.

Temp., 26-28°C; pH of water after flocculation, 6.3; dosage of aluminum sulfate, 100 ppm; estimated initial dose of virus in water, .025 LD_{50}/.02 ml; estimated amount of virus after redispersing, 1.0 LD_{50}/.02 ml.

pH of redispersing fluid*	Redispersing time, hr	Mice, infected /total	% mortality
7.5	1	1/11	9.1
	3	1/20	5.0
7.9	1	12/34	35.3
	3	9/34	26.4
8.5	1	12/37	32.4
	3	(4/7)†	
8.9	1	12/37	32.4
	3	8/23	34.7
Control made without alum at an estimated dose of .5 LD_{50}/.02 ml		15/52‡	28.8
		12/47§	25.5

* Bicarbonate buffer was used for pH 7.5 and carbonate buffer was used for the 3 higher pH values.

† No. of mice used was too small because of cannibalism in 3 cages.

‡ Control made after 1 hr standing.

§ " " " 3 " " .

following sets of conditions for each test number: 1. Different concentrations of alum within each redispersing time. 2. Redispersing times, averaging percent mortality over the 3 alum concentrations. 3. Alum concentrations, averaging over the 2 redispersing times. 4. All experimental conditions combined *vs.* control.

Finally, for the 3-hour redispersing time, average response after 60 and 80 ppm alum flocculation in both experiments was compared with the average response after 100 ppm alum.

The only clearly significant results were those comparing the over-all experimental averages with the controls. There was some indication, though not statistically significant, that 100 ppm alum gave somewhat better percent recovery of potency (mortality) than the other 2 concentrations. Therefore, the 1-hour redispersing time and 100 ppm alum were chosen for future experiments on a practical basis. On the average, sufficient virus was recovered to achieve about 70% of the potency (mortality) exhibited by the control.

Table II presents the results of an experiment designed to select the optimum pH for recovery of virus from flocs formed with 100 ppm of aluminum sulfate. $NaHCO_3$-HCl buffer at pH 7.5 was clearly not effective in separating virus. Analysis of the data on the remaining buffers by the chi-square test revealed no significant variation among percentage responses after separation at pH values of 7.9, 8.5, or 8.9 with 1-hour or 3-hour redispersing times. On the average, percentage mortality among mice inoculated with virus recovered from flocculation was 18% higher than that shown by the control material which had an estimated dosage of only 0.5 LD_{50} per 0.02 ml.

A precise estimation of percentage virus recovery cannot be given for these results unless a dosage-mortality curve is invoked, since percentage mortality is not always directly proportional to dilutions used. It may be stated, however, that a potency in excess of 0.5 LD_{50} but less than the theoretical 1 LD_{50} was recovered. This indicates, on a volume basis, that greater than 50% of potency was recovered.

The results of additional experimentation with the ion-exchange method are presented in Table III. Since the volume of resin required by this method is so large that it is difficult to collect the final supernate without contaminating it with resin, the concentration was reduced to 20 times so that twice the amount of supernate would be available. In these replicates the virus dosage in the beakers was doubled so that the final virus titer would be comparable with the levels in the 40-fold concentration.

When the percentage mortalities of these replicates are compared with the results ob-

TABLE III. Replicate Experiments with Ion-Exchange-Resin (Dowex I) Concentration Method.

Estimated initial virus titer, .05 LD_{50}/.02 ml
" final " " , 1.0 "

Mice, infected/total	% mortality
1/32	3.1
2/35	5.7
1/27	3.7
2/21	9.5

TABLE IV. Recovery of Coxsackie Virus from Water Concentrated 40 Times by Alum Flocculation Method.

Temp., 26-28°C; pH of water after flocculation, 6.3; dosage of aluminum sulfate, 100 ppm; pH of floc redispersing fluid, 7.9; estimated initial dose of virus in water, .00625-.025 LD_{50}/.02 ml; estimated amount of virus after redispersing, .25-1.0 LD_{50}/.02 ml; Redispersing time, 1 hr.

Estimated amt of virus per .02 ml of concentrate	Test No.	Mice, infected /total	% mortality
1 LD_{50}	1	16/80	20.0
	2	21/50	42.0
.5 "	1	10/51	19.6
	2	10/37	27.0
.25 "	1	2/100	2.0
	2	5/32	15.6
Controls made without alum at an estimated (Test 1) .625 LD_{50}/.02 ml (Test 2) 1.0 LD_{50}/.02 ml	1	12/47	25.5
	2	10/18	55.5

tained in the flocculation experiments in Tables I and II, it is obvious that the flocculation method is superior to the ion-exchange method in recovering virus activity.

Table IV shows the results obtained when water contaminated with lower amounts of virus was treated by flocculation. It will be noted that water containing as little as 0.00625 LD_{50}/0.02 ml can be flocculated, and that sufficient virus can be recovered to induce infection in 2 to 15% of the inoculated mice.

Discussion. This development of a sensitive and economical method of virus concentration fulfills a need which has hampered investigators in virus fields. Coxsackie virus was employed in these investigations because of its obvious sanitary significance as an enteric virus. Preliminary work on APC type 3 virus, not detailed here, has shown that virus also to be amenable to this concentration procedure, and it is possible that application of the flocculation method is feasible for detection of residual live virus in vaccines such as are now being produced for poliomyelitis immunization. Chang, *et al.*(3) reported much longer times were necessary for recovery of bacterial virus from the floc complex than we observed for Coxsackie virus. This may well represent inherent differences between the types of virus used in their work and the Coxsackie virus used in this work. Further investigation of these differences is in progress, particularly with respect to virus purification procedures.

Summary. A method for the concentration of Coxsackie virus from dilute suspension is described. This flocculation procedure is capable of detecting as little as 0.00625 LD_{50}/0.02 ml in the starting material and is shown to be more sensitive than an ion-exchange method currently used. It possesses the additional advantages of being economical and simple in execution.

We are indebted to Dr. E. K. Harris for the statistical analyses contained in this paper and to Mr. Richard A. Gallagher for competent technical assistance.

1. Melnick, J. L., Emmons, J., Opton, E. M., and Coffey, J. H., *Am. J. Hyg.*, 1954, v59, 185.

2. Kelly, S. M., *Am. J. Public Health,* 1953, v43, 1532.

3. Chang, S. L., Isaac, P. C. G., and Baine, N., *Am. J. Hyg.*, 1953, v57, 253.

4. Schaeffer, M., and Brebner, W. B., *Arch. Path.*, 1933, v15, 221.

5. Sabin, A. B., *J. Exp. Med.*, 1931, v56, 307.

6. Cochran, W. G., *Biometrics,* 1954, v10, 417.

Received July 2, 1956. P.S.E.B.M., 1956, v92.