Concentration of Reovirus and Adenovirus from
Sewage and Effluents by Protamine Sulfate
(Salmine) Treatment

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Protamine sulfate was employed to recover reoviruses, adenoviruses, and certain enteroviruses from sewage and treated effluents; 50- to 400-fold concentration of viral content was achieved.

The viral content of sewage fluctuates widely, being influenced not only by viral infection rates in the community at any given time, but also by hour of day, season of year, ratio of industrial to domestic waste, and extent of dilution by seepage or surface runoff into the sewerage system. Therefore, concentration of virus is required for an assay procedure sensitive enough to detect minimal quantities of virus in raw sewage and treated effluents.

The frequency of reovirus and adenovirus, in addition to enterovirus, in sewage of the San Diego area became apparent during a study in which the San Diego Public Health Laboratory participated from 1962 to 1965 (4). There are methods available for efficient concentration of viruses, but, during assay of the concentrated specimens, reoviruses and adenoviruses tend to be overgrown by more rapidly replicating enteroviruses. For this reason, a method that would preferentially concentrate reovirus or adenovirus over enterovirus was sought.

Chambers and Henle (1) and Warren et al. (12) reported precipitation of certain animal viruses by protamine sulfate. Cramer (3) stated that viruses of less than 50 nm in diameter remain in suspension during protamine treatment and those of larger diameter are precipitated, the precipitation reaction being reversible in 1 M NaCl. By this criterion, adenoviruses and reoviruses should be precipitated and enteroviruses should remain in suspension.

Studies in our laboratory established that protamine sulfate (salmine) treatment may be employed to concentrate adenoviruses and reoviruses; contrary to expectation, it also concentrated certain enteroviruses, whereas other enteroviruses remained in suspension. Furthermore, we found that bovine albumin added to sewage or effluent samples prior to salmine treatment permitted viral recovery that otherwise did not occur and exerted a sparing effect on the virus. Because bovine albumin is precipitated by salmine, the virus-salmine-albumin complex may result from a mechanism other than direct precipitation of virus by salmine. However, preformed albumin-salmine precipitate resuspended in water did not adsorb reovirus. The preformed precipitate resuspended in activated sludge effluent prior to addition of reovirus appeared to dissolve partially, and it yielded less than half the quantity of virus recovered when the precipitate formed in the presence of the virus.

Our procedure for concentration of viruses from sewage and partially treated effluents encompassed the following. All membrane prefilters (Millipore Corp.; no. AP20) were processed with Tween 80 to preclude adsorption of virus to the discs (11). Sample was prefilted through a double membrane disc (124 mm diameter; no. AP20, Millipore Corp.) held in a Buchner funnel. Sample volume varied from 200 to 2,000 ml. Bovine albumin was added to a final concentration of 0.25%. The albumin-supplemented sample was adjusted to pH 7.5 to 7.8 with 1 N HCl or 1 N NaOH. Salmine, as 1% stock solution, was added to final optimal concentration determined by prior titration (0.05% for first lot, 0.025% for second lot). The flask of reactants was placed on a magnetic stirrer for 30 min at room temperature. The precipitate was collected by passing the sample through a Millipore AP20 disc (47 mm diameter) held in a Millipore hydrosol stain-
less-steel filter holder. After vacuum filtration of sample, a 15-ml glass tube was attached below the outlet spout with the clamp provided. The precipitate on the upper surface of the AP20 disc was dissolved and collected in the test tube as follows. With the vacuum of 1 mNaCl (generally 0.5 ml) was pipetted over the surface of the AP20 disc, allowed to soak through for about 5 min, and then pulled into the receiving test tube by vacuum. Sterile water, six times the volume of 1 mNaCl, was pipetted over the prefilter disc and pulled through to rinse the disc and effect an isotonic filtrate. Fetal bovine serum, 10% by volume, was added to stabilize the virus. The concentrated suspension was centrifuged at 2,500 rev/min for 30 min to sediment bacteria, and the supernatant fraction was inoculated into cell cultures.

This procedure repeatedly recovered 80 to 100% of reovirus or adenovirus exogenously added to sewage. For recovery of naturally occurring virus in field samples of sewage and primary or secondary effluents, the procedure effected concentrations of 50- to 400-fold.

Experiments were performed to determine the fate of specific enteroviruses added to sewage and treated with salmine. Sufficient virus was added to insure that the inoculum would be diluted beyond the level of naturally occurring virus in the sewage. Different enteroviruses differed in their response. The polioviruses and coxsackieviruses tested were found in the supernatant fraction after centrifugation. Variable results were obtained with echoviruses. Six echovirus types were tested, with three consistently being recovered in the sediment. These were echovirus types 5, 6, and 11. Type 7 was almost entirely recovered from the supernatant fraction, and the greater portions of types 1 and 8 also were in the supernatant fraction.

For concentration of the viral content of 33 raw sewage, primary effluent, or secondary effluent samples, the salmine method was performed in parallel with one or more of the following adsorption-elution methods: Monsanto polyelectrolyte PE60 (9), Al(OH)₃, or CaHPO₄ (10). Selected results for raw sewage samples collected from two treatment plants over a 15-month period are shown in Table 1. On the average, the quantity of reovirus obtained by the salmine method exceeded that recovered by PE60 or CaHPO₄, and was at least comparable to that recovered by Al(OH)₃. Enteroviral recoveries by salmine relative to the other methods were variable, reflecting the differences we encountered among echoviral types tested with salmine. Adenoviruses, not recovered when the concentrated specimens were assayed in rhesus monkey kidney plaque tests, frequently were detected in most probable number tests (2) performed in tube cultures of human embryonic kidney cells and

### Table 1. Viral assay of raw sewage: reovirus and enterovirus recovered by different methods of concentration and assayed by plaque enumeration

| Source | Date | Salmine concn | Other concn |
|--------|------|---------------|-------------|
|        |      | PE            | Al(OH)₃     | CaHPO₄      |
|        |      | Reovirus      | Entero-virus| Reovirus   | Entero-virus| Reovirus | Entero-virus| Reovirus | Entero-virus|
| San Diego | 6/23/69 | 30<sup>a</sup> | 17 | 19 | 58 | 279 | 4 |
|          | 2/24/70 | 13 | 9 | <1 | 12 |      |      |      |
|          | 4/21/70 | 254 | 4 |      |      |      |      |      |
|          | 7/20/70 | 1,125 | <25 | 375 | <25 |      |      |      |
|          | 7/28/70 | 4,475 | <25 |      |      | 1,875 | <25 |      |
|          | 9/30/70 | 320 | 200 |      |      | 300 | 33 |      |
| Santee | 8/4/69 | 638 | 31 |      |      | 162 | 462 |      |
|         | 11/3/69 | 126 | <3 |      |      | 23 | 20 |      |
|         | 12/16/69 | 32 | <2 |      |      | <3 | <3 |      |
|         | 2/2/70 | <2 | 8 | <1 | 1 |      |      | 6,151 | <17 |
|         | 9/22/70 | 5,034 | <17 |      |      |      |      |      |

<sup>a</sup> Method of concentration: adsorption to and elution from Monsanto polyelectrolyte PE60 (PE), Al(OH)₃, or CaHPO₄; salmine treatment.

<sup>b</sup> Plaque-forming units per 100 ml of sample; rhesus monkey kidney cultures.
were predominant in 5 of 20 raw sewage samples concentrated by the salmon method and assayed for most probable number.

Reovirus has been recovered from sewage of San Diego area more consistently than any other virus; it has been reported only occasionally elsewhere in this country. However, studies from South Africa (7, 8), Germany (6), Poland (5), and the Netherlands (unpublished communication) have reported frequent recoveries of reovirus from sewage. Is its source human? Antibody surveys of people around the world have indicated its ubiquity. Many species of lower animals also are infected naturally by reoviruses, but their contribution to urban sewage must be relatively small. The extremely high titers of reovirus in some of our samples of sewage and the frequency with which we recover it from sewage throughout the year suggest that reovirus is endemic in the San Diego area and that individuals who are infected, probably subclinically, excrete large quantities into the sewage.

The presence of adenovirus in sewage has been recognized widely, but many studies have not encompassed methods suitable for its isolation. Until the complete role of reoviruses and adenoviruses in disease of man and lower animals is defined, they, as well as enteroviruses, should be sought in sewage and treated effluents. Protamine sulfate treatment provides an efficient method for concentrating reoviruses and adenoviruses, and its limited effectiveness for concentrating enteroviruses helps prevent overgrowth of reoviruses and adenoviruses by more rapidly replicating enteroviruses. Employment of this technique in parallel with any of several methods for concentrating enteroviruses permits an expanded knowledge of viruses recoverable from sewage.

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