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Reduction of Interfering Cytotoxicity Associated with Wastewater Sludge Concentrates Assayed for Indigenous Enteric Viruses

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Washing, freon extraction, and cationic polyelectrolyte precipitation were compared for their ability to reduce cytotoxicity associated with virus concentrates derived from beef extract eluates of wastewater sludges. Eluates concentrated by hydroextraction were usually much more toxic than those concentrated by organic flocculation. This difference may be due entirely to nondialyzable material naturally present in the beef extract which did not precipitate during flocculation at pH 3.5. Washing inoculated cell monolayers with saline containing calf serum before the addition of agar overlay media was most effective in reducing cytotoxicity, although it resulted in a greater virus loss, as compared with freon extraction and cationic polyelectrolyte precipitation.

The fate of human enteric viruses is one of the major public health concerns associated with handling and disposal of wastewater sludges. It is important, therefore, to have available reliable and simple methods for monitoring viruses in sludges. In response to this need, a variety of methods have been proposed. These are the subject of a soon-to-be-published review (C. J. Hurst. 1984. Recovering viruses from sewage sludges and from solids in water, *in* G. Berg (ed.), *Viral Pollution of the Environment*, CRC Press, Inc., Boca Raton, Fl.). The most promising methods utilize beef extract solutions for elution of adsorbed viruses from sludge solids (1, 11).

The quantities of viruses contained in wastewater sludges are sometimes relatively low, as is often the case with viruses collected from environmental samples. For this reason, as well as the considerable expense involved in performing virological analyses on large volumes of sample material, processed sludge eluates are often subjected to concentration procedures before assay. Organic flocculation as described by Katzenelson and co-workers (6) and hydroextraction against polyethylene glycol as described by Wellings and co-workers (11) are the methods most commonly chosen for use in concentrating of viruses from beef extract eluates.

Unfortunately, cytotoxicity of substances present in the concentrated samples is often severe enough to interfere with accurate virus quantitation. The toxicity may be due to metals and/or organics which originate in the sludge or in the reagents. One solution is simply to dilute the concentrated material before it is introduced into cell culture systems. However, this practice acts to offset the cost benefits concentration provides. This is particularly important in monitoring programs in which it is necessary to assay a sufficient portion of each sample such that the numerical data obtained will be statistically meaningful.

The purpose of this work was to coevaluate the organic flocculation (6) and hydroextraction (11) methods for concentrating viruses eluted from wastewater sludges, along with three different techniques for reducing cytotoxicity associated with the produced concentrates. The techniques examined for reducing sample toxicity were treatment of concentrates by (i) freon extraction (L. M. Stark, F. M. Wellings, and A. L. Lewis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q51, p. 209); (ii) addition of a cationic polyelectrolyte, Cat-Floc T (D. A. Wait, H. P. Kimball, and M. D. Sobsey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q41, p. 207); and (iii) washing inoculated monolayers with saline containing calf serum immediately before the addition of overlay medium. General detoxification techniques have received foremost consideration for this purpose, as the exact nature of chemical cytotoxicants present in processed sludge samples remains unknown.

During the course of this study, a series of experiments was conducted to determine whether reagent-related factors contributed to the increased cytotoxicity associated with hydroex-

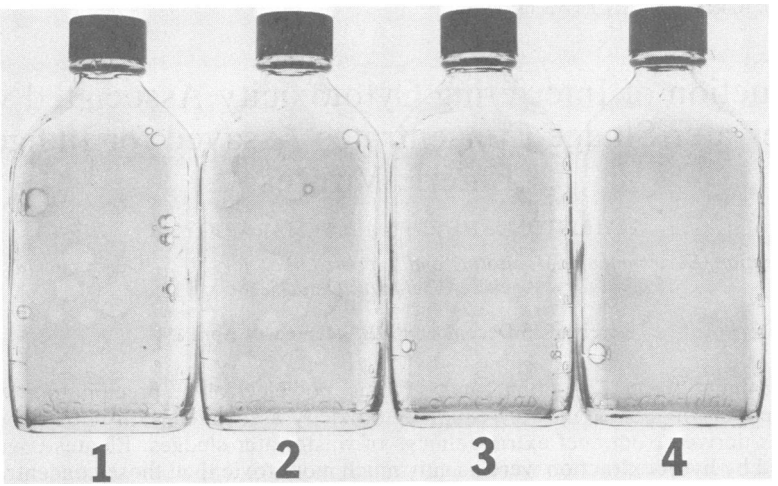

FIG. 1. Cytotoxicity ratings of cell monolayers inoculated with environmental samples. From left to right, the bottles show increasing levels of toxicity. In this case, the bottle rated 4 contains no visible remains of a cell monolayer.

traction concentrates. An attempt was also made to reduce the cytotoxicity of hydroextracted samples by passing them through a positively charged depth filter (5).

MATERIALS AND METHODS

Sewage. Sewage samples were obtained from Millcreek Wastewater Treatment Plant and Muddy Creek Sewage Works, both of which are located in the Cincinnati, Ohio, area. The samples consisted of either settled primary sludge solids or whole mixed-liquor activated sludge. The influent at the Millcreek Plant receives substantial amounts of industrial effluent, whereas the Muddy Creek Plant receives relatively little. Sludge was collected by grab sampling, using sterile autoclavable plastic containers. Processing of each sample was begun within 90 min of collection. Samples were collected over a 1-year period at intervals of approximately 2 weeks. Five primary and six activated sludge samples were collected from each of the two plants. Since the objective of the study was to compare overall effectiveness of toxicity reduction methods, the data were not segregated with regard to sample origin.

Virus recovery and assay. All studies utilized only those viruses indigenous to the sludge samples. The technique used for elution of virus from the sludge solids was basically that developed in this laboratory and described by Berman and co-workers (1). Samples (200 ml each) of sludge were adjusted to pH 3.5 and supplemented with aluminum chloride at a final concentration of 0.0005 M by the addition of 1 N hydrochloric acid and 0.05 M aluminum chloride, respectively. The samples were then mixed for 30 min on a magnetic stirrer and centrifuged for 15 min at 1,350 × *g* to pellet the sludge solids. The pelleted solids from each sample were suspended in 200 ml of a 10% (wt/vol) solution of beef extract (beef extract purchased in powdered form from GIBCO Diagnostics, Madison, Wis., and Oxoid Ltd., Long, England) containing 1.34% (wt/vol) $Na_2HPO_4 \cdot 7H_2O$ and 0.12% (wt/vol) citric acid. The resulting mixtures were stirred for 30 min and centrifuged for 30 min at 7,000 × *g* for collection of the elution supernatant (eluate). The eluate was then passed through a filter (pore size, 0.22 μm) (Millipore Corp., Bedford, Mass.) to remove bacteria.

The resulting filtrates were concentrated by either organic flocculation (6) or hydroextraction (11). Paired samples of concentrates were produced by dividing the filtered eluate obtained from each sludge sample into two equal 100-ml portions; one portion was then processed by each of the two concentration techniques. As the organic flocculation method of Katzenelson and co-workers was designed to concentrate 3% solutions of beef extract, samples of sludge eluates to be processed by this method were diluted 1:3.3 with sterile distilled water (final effective beef extract concentration, 3.0%) before concentration. Organic flocculation was accomplished by adjusting the diluted sludge eluate to pH 3.5, stirring the eluate for 30 min, and collecting the resulting floc by centrifugation for 10 min at 1,350 × *g*. The floc was dissolved in 5 ml of 0.15 M Na_2HPO_4 buffer solution per 100 ml of diluted eluate concentrated and then assayed. Hydroextraction of filtered sludge eluates and beef extract control samples was done by using polyethylene glycol (average molecular weight, 15,000 to 20,000; Matheson, Coleman & Bell, Norwood, Ohio). Each sample was hydroextracted until the minimum attainable volume was reached (endpoint hydroextraction). The volume at endpoint hydroextraction was approximately 20 ml for each 100 ml of 10% beef extract concentrated. During hydroextraction, the samples being concentrated were contained in dialysis tubing (12,000 molecular weight cutoff), the ends of which were sealed with plastic dialysis tubing clamps (both dialysis tubing and clamps purchased from Arthur H. Thomas Technological Service, Philadelphia, Pa.). When paired samples of sludge concentrates were prepared for cytotoxicity comparison, the concentrates from the two procedures

TABLE 1. Percentage of trials in which sludge eluate concentrates could be assayed without dilution

Concn procedure[a]	% of trials[b]	
	Untreated	Washing
Organic flocculation	82 (9/11)	100 (11/11)
Hydroextraction	<25 (0/4)	<25 (0/4)

[a] Six of the eleven organic flocculation concentrates tested were produced from activated sludge; the other five were from primary sludge. The hydroextraction concentrates represent two samples of activated sludge and two of primary sludge.

[b] Numbers in parentheses are the ratios of those concentrates tested which produced cytotoxicity ratings of 1 or 2 to the total number of concentrates examined.

were made equal in volume by the addition of sterile saline to that concentrate having the lesser volume. Viruses were assayed in 6-oz. flint glass prescription bottles by using the BGM continuous cell line and a soft agar overlay plaque technique. The procedures used for culturing the BGM cell line and for performing plaque assays have been described previously by Dahling et al. (3).

Toxicity reduction. When inoculated monolayers were treated by washing, this was done after the sample was allowed to incubate on the monolayers for 1 h at room temperature so that virus adsorption to the cells could occur. After this exposure period, the inoculum was poured out of the cell culture bottles and replaced with 10 ml of 0.85% (wt/vol) sodium chloride containing 2% (vol/vol) calf serum. This saline solution was gently rocked twice across the monolayers and poured off, and soft agar overlay medium was added to the bottles.

Concentrates treated with freon were first chilled in an ice bath; then an equal volume of Freon TF (1,1,2trichloro-1,2,2trifluoroethane) was added (Freon TF provided by E. I. du Point de Nemours & Co., Inc., Wilmington, Del.). The mixtures were homogenized by vigorous shaking (five complete inversions of the mixture per second) for 30 s and subsequently centrifuged at 1,200 × *g* for 30 min. The resulting aqueous phases were aspirated and assayed.

Concentrates treated by cationic polyelectrolyte precipitation were adjusted to a final concentration of 0.1% (vol/vol) Cat-Floc by the addition of a 1.0% (vol/vol) aqueous stock solution of Cat-Floc T (Cat-Floc T provided by Calgon Corporation, subsidiary of Merck & Company, Inc., Pittsburgh, Pa.). The sample-Cat-Floc mixtures were agitated at 20 rpm on a rotary shaker for 20 min and subsequently centrifuged at 1,850 × *g* for 10 min to remove any resulting precipitate. The supernatants produced from Cat-Floc treatment of samples were then assayed, and the virus titers were adjusted upward by a factor of 10% to correct for dilution caused by the addition of Cat-Floc stock solution.

Heavy metals analysis. Samples of unconcentrated and concentrated beef extract solutions were analyzed for the presence of cadmium, zinc, and lead by means of atomic absorption spectrophotometry.

Evaluation of cytotoxicity. The inoculated monolayers were examined for cytotoxicity after incubation for 4 days at 37°C. Based on their visual appearance, each culture was grouped into one of the following categories.

(i) Rating category 1. Monolayer greater than 95% intact, pink in color, and very healthy in general appearance. Plaques well shaped and easily identifiable, with plaque edges sharply distinguishable from surrounding monolayer.

(ii) Rating category 2. Monolayer 90 to 95% intact and generally pink in color, although somewhat unhealthy or "dull" in overall appearance. Plaques still easily identifiable, and plaque edges distinguishable from the surrounding monolayer.

(iii) Rating category 3. Monolayer approximately 40 to 90% intact, frequently yellow to yellow-brown in color, and generally very sparse or "faint" in appearance. Plaques difficult to distinguish from surrounding monolayer, with plaque edges being very "diffuse."

(iv) Rating category 4. Less than 40% of monolayer remaining and usually yellow to yellow-brown in color. Individual plaques not usually discernable.

For each day on which cell monolayers were evaluated, standards were selected for each of the four cytotoxicity ratings. All other cell culture monolayers to be examined on that day were then assigned cytotoxicity ratings of 1 through 4 based on comparison with those cell culture monolayers selected as standards. As a necessary precaution, all of the cell culture monolayers to be inoculated and subsequently compared as a group for the presence and extent of cytotoxicity were of the same cell culture passage lot. An example of inoculated cell monolayers fitting the four cytotoxicity ratings is presented in Fig. 1. All four plaque assay bottles shown were inoculated on the same day but with different virus samples. Inoculated monolayers rated in categories 1 and 2 yielded viral assay titers that were reproducible, and titers of different dilutions of these samples were numerically equivalent. Viral titers obtained from assays in which the inoculated monolayers fit into rating category 3 were inconsistent, with titers from reassays of the same samples differing by as much as 1.5 orders of magnitude. Therefore only those samples for which the inoculated monolayers fit into rating categories 1 or 2 were considered to be assayable. All samples evaluated for cytotoxicity were inoculated at dilutions of 1:1 (undiluted), 1:2, and 1:5. The samples were diluted in 0.85% (wt/vol) sodium chloride containing 2% (vol/vol) calf serum. All inoculations were done in duplicate. In instances in which the toxicity ratings of duplicate monolayers differed, the least toxic rating was arbitrarily recorded as the cytotoxicity value.

RESULTS

Unconcentrated virus eluates produced from sludges in this laboratory by the technique of Berman et al. (1) have not been found to be cytotoxic. However, concentrates produced from these same eluates are quite frequently cytotoxic. The goal of this study was to determine whether the cellular destruction caused by cytotoxicants present in processed virus concentrates could be reduced by removing the

TABLE 2. Percentage of trials in which sludge eluate concentrates could be assayed without dilution

Concn procedure[a]	% of trials[b]			
	Untreated	Washing	Freon extraction	Cat-Floc precipitation
Organic flocculation	82 (9/11)	91 (10/11)	64 (7/11)	82 (9/11)
Hydroextraction[c]	<11 (0/9)	100 (9/9)	<11 (0/9)	44 (4/9)

[a] Six of the pairs of sludge eluate concentrates were produced from activated sludge and five from primary sludge.

[b] Numbers in parentheses are the ratios of those concentrates tested which produced cytotoxicity ratings of 1 or 2 to the total number of concentrates examined.

[c] Results from two of the hydroextraction concentrates were not included because of heavy microbial contamination.

virus concentrate from inoculated cell monolayers after virus adsorption, before the addition of agar overlay media. Data from the first set of 11 virus concentrates (representing samples collected, processed, and assayed over a 6-month period) shows that the washing procedure appears to offer an advantage with regard to viral assay of sludge eluates concentrated by the organic flocculation procedure (Table 1). Use of the washing procedure also reduced the toxicity of virus eluates concentrated by hydroextraction, although not sufficiently to permit these concentrates to be assayed undiluted.

An evaluation was then made of two additional methods which have been proposed for reducing sample-associated cytotoxicity: freon extraction and cationic polyelectrolyte precipitation. These methods were compared to the monolayer washing procedure by using a second set of 11 pairs of virus concentrates prepared from sludge samples collected, processed, and assayed over a subsequent 6-month period. Each sample of concentrate to be assayed was divided into four portions. One portion of each sample was assayed without treatment (untreated). Each of the three remaining portions was assayed in conjunction with one of the three toxicity reduction treatments. The results are presented in Table 2.

Of the three methods, monolayer washing was the most effective for reducing sample cytotoxicity. Virus eluates concentrated by organic flocculation were again generally less cytotoxic than those concentrated by hydroextraction. Observed differences in effectiveness of the three toxicity reduction techniques were relatively slight when evaluated in terms of their ability to increase the overall extent to which organic flocculation concentrates could be assayed. These slight differences do, however, appear to be reproducible when compared with those presented in Table 1, which were obtained from an entirely different group of sludge samples. The two organic flocculation concentrates listed in Table 2 as being unassayable when undiluted and with no toxicity reduction treatment are the same two which were unassayable without dilution after Cat-Floc precipitation and were two of the four organic flocculation concentrates which were unassayable without dilution after freon extraction. Only one of these two concentrates was unassayable without dilution by the washing procedure.

From the data presented in Table 2, it would appear that freon extraction might have increased the cytotoxicity of the organic flocculation concentrates. Two control samples of organic flocculation concentrates which had been produced from sterile solutions of 10% beef extract were assayed both with and without exposure to the various toxicity reduction treatments, and in all cases the inoculated monolayers had a toxicity rating of 1.

As shown in Table 2, differences in effectiveness among the three toxicity reduction treatments were much greater with concentrates produced by hydroextraction. None of the nine hydroextraction concentrates tested could be assayed undiluted either without treatment or after freon extraction. With washing, however, all nine hydroextraction concentrates could be assayed without dilution. Four of the nine samples could be assayed undiluted if treated by Cat-Floc precipitation.

In an overall summary of the data presented in Tables 1 and 2, 18 of 22, or approximately 82%, of sludge eluate concentrates produced by organic flocculation could be assayed undiluted without toxicity reduction treatment. Of the remaining four untreated organic flocculation concentrates, one could be assayed at a 1:2 dilution, two could be assayed at a 1:5 dilution, and one would have required a dilution greater than 1:5 to be assayable. With the use of the monolayer washing procedure, the degree of assayability of the undiluted organic flocculation concentrates was improved to 21 of 22, or approximately 95%. In general, a 1:2 dilution was sufficient to allow assay of all of the organic flocculation concentrates when any of the three

TABLE 3. Decrease in virus titer caused by toxicity reduction treatments[a]

Type of treatment	Titer reduction (%)
Washing	28 ± 22
Freon extraction	20 ± 13
Cat-Floc precipitation	6 ± 22

[a] Percent reduction in virus titers of treated eluate concentrates based on titers of identical, untreated samples. The percent decreases in virus titer are the overall averages of the percent decreases observed in all experiments ± 1 standard deviation.

toxicity reduction treatments was utilized.

Hydroextraction concentrates showed no cytotoxicity after washing (Table 2). In this regard, the findings reported in Tables 1 and 2 differ. The materials and techniques used in these two sets of experiments were identical. This variation in toxicity simply reflects natural differences among the groups of samples owing to their having been collected over two separate time periods. The findings in these two tables have been combined in the summary below to estimate the overall assayability of hydroextracted sludge eluates. Without toxicity reduction treatment, none of the 13 hydroextracted samples was assayable without dilution. Nine of the untreated hydroextraction concentrates could be assayed at a 1:2 dilution, and the remaining four could be assayed at a 1:5 dilution. With the monolayer washing technique, the overall assayability of these samples, without dilution, was 9 of 13, or approximately 69%. The remaining four washed samples were assayable at a 1:2 dilution. The overall assayability rate for undiluted, Cat-Floc-treated hydroextraction concentrates was 44%. Each of the five Cat-Floc-treated hydroextraction concentrates which were unassayable without dilution was assayable at a 1:2 dilution. None of the nine freon-treated hydroextraction concentrates could be assayed undiluted. Of these nine concentrates, four were assayable at a 1:2 dilution, and the remaining five could be assayed at a 1:5 dilution.

The qualitative differences in the appearance of the cell monolayers resulting from the three toxicity reduction methods were more striking than would be expected from the small differences in the data in Tables 1 and 2. A visual comparison of cell monolayers inoculated with identical dilutions of a cytotoxic virus concentrate, both with and without toxicity reduction treatments, is presented in Fig. 2. For this particular sample, only the washed monolayer (B) is considered assayable, with a toxicity rating of 2.

During the course of these investigations, each of the three toxicity reduction treatments was found to cause a reduction in the virus titers of concentrates produced by both the hydroextraction and organic flocculation techniques. The extent of titer reduction was similar with washing and freon extraction treatments and lower with Cat-Floc treatment (Table 3).

A series of experiments conducted to determine whether reagent-related factors contributed to the increased cytotoxicity associated with hydroextraction concentrates showed no difference in the extent of cytotoxicity among hydroextraction concentrates produced from beef extract solutions prepared either with or without buffering salts; beef extract solutions prepared in glassware previously cleaned with only distilled water versus those prepared in detergent washed glassware; or upon treatment of dialysis tubing by boiling in and rinsing with either saline or distilled water before use in hydroextraction. Extensive dialysis of hydroextracted beef extract solution against physiological saline, followed by vacuum dialysis to reduce the resultant increase in sample volume, had no apparent effectiveness in reducing toxicity.

Based on the volume of beef extract solutions remaining after endpoint hydroextraction, the final concentrates were approximately equivalent to a 50% solution of powdered beef extract. The toxicity of 50% beef extract alone was approximately equal to that of hydroextraction concentrates. Organic flocculation is principally a selective fractionation procedure, whereas hydroextraction nonspecifically increases the concentration of all substances retained by the dialysis tubing. In an effort to determine why the hydroextraction concentrates were so much more cytotoxic than the organic flocculation concentrates, samples of the supernatant produced during organic flocculation of beef extract solutions were collected and hydroextracted. The resulting concentrates were then diluted with saline to volumes equal to those resulting from directly hydroextracting equivalent amounts of 10% beef extract. These concentrated supernatants were extremely cytotoxic, indicating that the differences in toxicity between concentrates produced by the two techniques were due at least in part to nondialyzable material naturally present in the beef extract which remained soluble at pH 3.5.

Atomic absorption spectroscopy of beef extract solutions indicated concentrations of less than 0.05 to 0.14 mg of cadmium per liter, less than 0.1 mg of lead per liter, and 0.7 to 1.2 mg of zinc per liter. Hydroextraction did not appreciably change the concentrations of these metals. Thus, heavy metals did not appear to be primarily responsible for the increased cytotoxicity

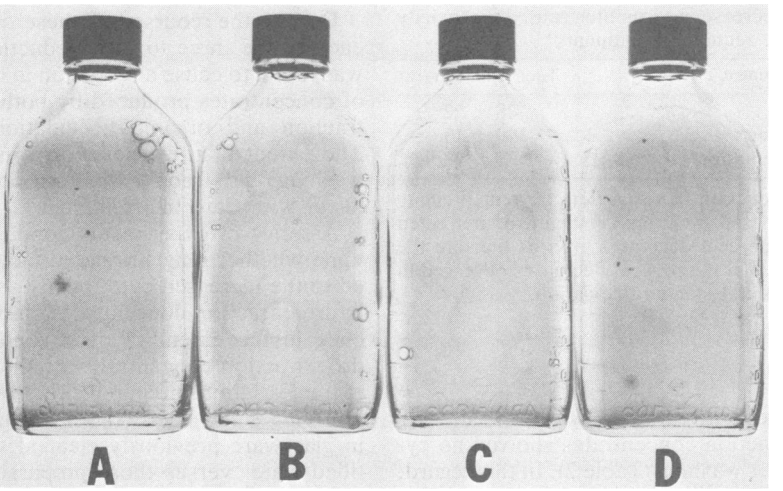

FIG. 2. BGM cell monolayers inoculated with a cytotoxic sludge eluate concentrate. Bottle A received no toxicity reduction treatment. Bottles B, C, and D were treated by washing, freon extraction, and Cat-Floc precipitation, respectively. No evidence of a cell monolayer remains in bottle A; thus, it was given a toxicity rating of 4. Bottle B was assigned a cytotoxicity rating of 2 and is the only one of the four considered assayable. Bottles C and D were both assigned cytotoxicity ratings of 3. Only part of the cell monolayer remains evident in bottle C. Bottle D still appears to contain a very faint monolayer; however, its overall degree of intactness is not sufficient to permit individual plaques to be clearly discerned.

associated with the hydroextraction concentrates, although a synergistic contribution to toxicity could not be ruled out.

Attempts to reduce the intrinsic cytotoxicity of hydroextraction-concentrated beef extract solutions by passing them through a positively charged depth filter (Zeta-plus; type 50s), a procedure reported by Hejkal et al. (5) to reduce the cytotoxicity of organic flocculation concentrates produced from samples of wastewater and sediment, did not show this technique to be effective.

DISCUSSION

To accurately evaluate techniques for alleviating the cytotoxic effects of virus concentrates from sludge samples on viral assay systems, it is necessary to develop simple methods for assessing this type of toxicity. The cytotoxicity scoring method developed during the course of this study satisfactorily fulfills this need. Before concentration, all the virus eluates from sludge samples examined were assayable without need for either dilution or use of toxicity reduction treatments. Concentrates produced from these sludge eluates were frequently cytotoxic. The degree of toxicity associated with concentrates produced by hydroextraction was much greater than that associated with concentrates produced by organic flocculation. Nondialyzable substances which were present in the beef extract and which did not precipitate at pH 3.5 may have been responsible for all of the increased toxicity associated with the hydroextracted samples. Other possible factors which were ruled out as directly causing the cytotoxicity of the hydroextracted samples were buffering salts used in preparing beef extract eluants, detergent residues on glassware used to process samples, water-soluble compounds associated with the dialysis tubing, substances contained in the polyethylene glycol which might have passed through the dialysis tubing during the course of the hydroextraction procedure, and increased levels of heavy metals present in the concentrates. Because heavy metals did not appear to have caused the cytotoxicity associated with concentrated samples, the method proposed by Glass et al. (4) for reducing toxicity due to heavy metals was not evaluated in this study.

Although the hydroextraction concentrates studied were prepared from 10% solutions of beef extract, as compared to the 3% solutions used by others (11), this difference is unimportant inasmuch as endpoint hydroextraction should theoretically reduce both initial concentrations of beef extract solution to a common final concentration.

Until the matter of inherent cytotoxicity associated with hydroextraction concentrates produced from beef extracts can be resolved, the use of this method of concentrating virus should be avoided. Development of techniques for fractionating beef extract solutions before use in

virus studies might be helpful in overcoming this toxicity. The only work currently known on this subject is that of Berman et al. (2), which showed that most of the virus elution capacity of beef extract solutions remained in the supernatant fraction after low pH organic flocculation. Although such supernatants would also contain the cytotoxic material, it may prove possible to further treat the supernatants in such a way as to eliminate the cytotoxic material without affecting virus elution capability.

Washing of cytotoxic material from cell culture monolayers is a generalized procedure intended to remove from the surface of the cells any cytotoxic compounds which are neither taken up by nor tightly bound to the cells during incubation with virus sample inoculum. The particular method used in this study was designed to minimize any possible harsh effects on the cells. The freon extraction and cationic polyelectrolyte precipitation procedures were chosen for inclusion in this study based on their previous applications for removal of cytotoxic materials from environmental samples. The rationale behind the use of freon extraction has been that lipophilic compounds present in the sample will partition into the hydrocarbon layer, and extraneous protein impurities will denature and collect at the interphase between the hydrocarbon and aqueous layers (7–9). Cationic polyelectrolyte has been used on the premise that it may result in the removal of interfering surface-charged compounds through coprecipitation, the principles of which have been summarized by Thomson and Foster (10). Some sample toxicity was removed by the latter procedure, although this removal was more limited than that achieved through washing.

All three methods examined for toxicity reduction were detrimental in that they reduced virus titer. The most likely source of virus loss associated with the cell monolayer washing procedure was removal of virions from the cell surface before initiation of infection. The most likely causes of virus titer reduction due to freon extraction were by direct denaturation of virion proteins or through association of virions with the material which collected at the interphase between the hydrocarbon and aqueous layers. Reextractions of the freon phase were not performed because of the large increase in total sample volume which would have resulted. The most likely cause of virus losses resulting from Cat-Floc treatment is association of virions with the precipitate produced.

For virus environmental monitoring programs to operate with maximum effectiveness, it is important to either avoid or surmount problems such as sample- or reagent (method)-associated cytotoxicity. Although the levels of virus loss associated with the three toxicity reduction treatments evaluated cannot be ignored, in virus monitoring programs the benefit derived from use of these treatments outweighs this amount of loss. The only alternative would be to dilute the virus samples, which would result in an increased cost of assays or necessitate a reduction in the number of samples tested.

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