In summer and fall months the Coxsackie viruses may frequently be recovered from municipal sewage treatment plants when laboratory methods described here are employed.

Detection and Occurrence of Coxsackie Viruses in Sewage*†

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SEWAGE and water supplies, long recognized as vehicles for bacterial agents of disease, are now under suspicion as carriers of viruses as well. The virus of poliomyelitis, for example, has been isolated from sewage [1-5] and from water [6]; hepatitis virus in the water supply has been indicted as the cause of outbreaks [7]; and Coxsackie virus recoveries from sewage have been reported.[8, 9] These observations raise the questions: how frequently do viruses occur in sewage and water during normal times, and how well, if at all, do they survive standard water sanitation practices? Such information can best be obtained by the use of a simple, rapid, sensitive, and safe method for detecting viruses. This report presents such a method and the results of testing under field conditions.

The method, in brief, consists of virus adsorption on, and elution from, ion-exchange resins. While Amberlite (Rohm and Haas Company) ion-exchange resins have been used previously to purify,[10] and also to concentrate virus preparations from infected tissue suspensions,[11-13] the procedures to be described employ other resin types and illustrate their use in recovering viruses that occur in sewage in amounts undetectable without special treatment.

DETECTION

A preliminary survey of methods that might be adapted to virus detection in water, including protamine, calcium phosphate, and zinc glycine precipitation, ion-exchange, and Sharples centrifugation, indicated that ion-exchange technics possessed peculiar advantages. Conditions for optimum virus recovery from sewage using ion-exchange resins were determined, therefore, for Theiler (T.O.) virus and *Escherichia coli* B bacteriophage. The better methods were then tested with strains of Coxsackie viruses. The procedure described below was found most satisfactory for detecting unknown strains of Coxsackie viruses in sewage.

To sewage samples, 60–100 ml. in volume, 30 per cent solution of bovine albumin (Armour) was added to give a final albumin concentration of 0.5 per cent. Dowex 1 resin, 200–400 mesh, 10 per cent cross-linkage, was added to the sewage in the proportion of 10 gm. per 100 ml. of sewage, and mixed for 3–4 minutes. The suspension was centrifuged at 2,500 r.p.m. for 10 minutes and the supernate discarded. To the sedimented resin was added 1 or 2 ml.

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TABLE 1

Recovery of Theiler Virus on Ion-Exchange Resins (Volumes of the Various Fractions Are the Same for Any One Set of Resin Data)

A. Fraction	M.E.D.* (neg. log)				
	Dowex 1 10 per cent			XE-67B	Nalco SAR
	(1)	(2) †	(3) †		
Original suspension	6.0	7.3	6.1	6.3	6.2
Supernate	3.7			<3	<2
Wash	2.0			<2	
Eluate	6.6	8.9	7.7	5.6	4.8

B. Fraction	Infected/total animals					
	Dowex 1 2 per cent	XE-64	Nalco		Dowex	
			HCR	SAR	50	1
Original suspension 10^{-5}	5/8		8/8	8/8		
Supernate 10^{-2}		8/8	3/3	6/8	7/8	1/7
Eluate undiluted	1/8	9/9	0/8	7/7	0/8	8/8

* M.E.D. determined by the Thompson method of moving averages [14]
† Suspensions first passed through bead-size resins, Amberlites IR-120, and IRA-410, and adsorbed on Dowex 1 twice

of 10 per cent disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$), the phosphate and resin mixed for 5–10 minutes, and the mixture centrifuged at 3,000 r.p.m. for 15 minutes. The eluate was drawn off, saved, and treated with antibiotics, 500 units of penicillin and 2½ mg. of streptomycin per ml. of eluate. These preparations were kept for convenience at 8°–10° C. overnight before being injected intraperitoneally and subcutaneously into the test animals, one- and two-day-old mice of the Albany standard strain. The development of limb paralysis or spasticity during a two-week observation period was taken as presumptive evidence of the presence of Coxsackie virus. Confirmation was made by passage of brain or leg material from such infected mice into healthy suckling mice and by its failure to induce evident disease in older (10–12 gm.) mice inoculated intracerebrally. Microscopic examination for typical lesions was also carried out. Serologic identification of many of the isolations was made by neutralization test with antisera from known strains of Coxsackie viruses.

Among the conditions found to influence virus recovery from sewage, were the following:

Resin Choice

Experiments with concentrated tissue suspensions and with dilute water and sewage suspensions of Theiler-virus-infected mouse brain indicate that of the resins tested, Dowex 1, 10 per cent cross-linkage, 200–400 mesh, is most satisfactory for virus recovery (Table 1). By preliminary passage of virus suspensions through bead-size resins, Amberlites IR-120 and IRA-410, and readsorption on the fine-particled resin as recommended by Lo Grippo and Berger,[12] recovery of virus was enhanced. Amberlite XE-67B, an anionic-exchange resin (as is Dowex 1) adsorbs Theiler virus; the yields, however, are not so quantitative as with Dowex 1, nor can the resin-sewage suspensions be transferred so cleanly from one vessel to another. The cationic-exchange resins, Amberlite XE-64 and Dowex 50, do not adsorb Theiler virus quantitatively from tissue suspensions. The Nalcite resins, SAR and HCR, similar to Dowex 1 and 50, respectively, only of larger particle

TABLE 2

Albumin Effect on Theiler Virus Recovery on Ion-Exchange Resins

Fraction		Infected/total animals				M.E.D. (neg. log)			
		Amberlite XE-67B		Dowex 1		Dowex 1			
		Albumin Absent	Albumin Present	Albumin Absent	Albumin Present	Vol. (ml.)	Albumin Absent	Egg Albumen	Bovine Albumin
Original suspension	10^{-5}	7/10	6/8	7/8	8/8	50	7.9	7.9	7.9
Eluate	undiluted	0/8	3/8	0/8	7/8	1	1.0	8.2	7.2

size, when ground to 200–400 mesh size, adsorb Theiler virus according to their corresponding Dowex resins. Dowex 1 resin, 2 per cent cross-linkage, does not adsorb satisfactorily.

Albumin Concentration

While the addition of albumin is not necessary for adsorption of virus from concentrated tissue suspensions, virus in dilute water or sewage suspensions is not recovered unless albumin is added (Table 2). This technic of protein addition was used successfully by Gard [15] in detecting poliomyelitis virus by ammonium sulfate precipitation. Recovery is equally good when the source is 30 per cent bovine albumin solution, powdered bovine albumin, or egg albumen. The final albumin concentration is not critical, 0.1–2.0 per cent giving similar recoveries.

Sewage Sample

When sewage samples for analysis are taken at one time or at stated intervals, as they frequently are, the sample is representative only of the sewage taken at that instant. Although this time limitation may not be important in certain analyses, it should not be ignored in the detection of agents whose presence in sewage may be intermittent, such as the Coxsackie viruses. Twenty-four and 48-hour sewage samples, collected as described below, therefore, were compared for their Coxsackie-virus content with catch sewage samples taken either at the beginning or end of the 24- to 48-hour period. The 24- to 48-hour samples were obtained by exposing cheesecloth swabs to the flow of sewage for the desired time and expressing the liquid. This swab procedure is essentially the same as that originated by Moore [16] and used successfully in locating typhoid carriers [17] and sources of poliomyelitis virus.[18] Such swab expressions yield Coxsackie viruses more consistently than catch samples of sewage (Table 3). When these swab

TABLE 3

Comparison of Sampling Methods on Coxsackie Virus Recovery from Colonie Sewage Treatment Plant

Date of Collection	Sample	Infected/Total Animals
9/11/52	Catch	0/16
9/8–9/11/52	Swab	3/16
9/11/52	Catch eluate	0/16
9/8–9/11/52	Swab eluate	8/16

expressions are coupled with resin treatment, the frequency of recovery is greater also than from similarly treated catch samples (Table 4). The use of

TABLE 4

Comparison of Sampling Methods and Resin Treatment on Frequency of Coxsackie Virus Recovery from Sewage

Sample	Total Number of Sewages	Positive Sewages	Per cent Positive Sewages
Catch	16	2	12.5
Swab	8	4	50
Catch eluate	19	11	58
Swab eluate	14	10	71

resin-treated swab expressions detected virus more consistently throughout the period tested than other sampling procedures (Table 5).

OCCURRENCE

In order to test the procedure de-

TABLE 5

Comparison of Sampling Methods on Frequency of Coxsackie Virus Recovery from Sewage During Different Months

Sample	Positive/Total Sewages			
	August	*September*	*October*	*November*
Catch	0/9	2/7		
Swab	2/3	2/2	0/3	
Catch eluate	5/10	6/9		
Swab eluate	2/3	1/1	6/9	2/8

scribed above under practical conditions, sewages in the Albany area were examined from July, 1952, through March, 1953, for the presence of Coxsackie viruses.

TABLE 6

Sewage Treatment Plants Tested for Coxsackie Viruses

	Estimated Population Served (Thousands)	*Plant*
+	130	Albany
	50	Schenectady
	15.2	Watervliet
	9	Delmar
	7.8	Scotia
	5	Rotterdam
	3	Colonie
−	2.6	Latham
	2	Ravena

Locality

During a one-month period, July 28 to September 3, raw sewage from nine treatment plants within 20 miles of Albany was sampled. Of these, seven contained Coxsackie viruses. The two plants where virus was not recovered were the smallest examined, serving a small population (Table 6). From data given below (Figure 1), this testing interval falls within the period of greatest incidence of Coxsackie viruses in sewage; it is probable, then, that if the viruses occurred in the locality, they would appear during this interval or not at all.

Seasonal Incidence

Three of the plants where Coxsackie viruses were detected initially in August, 1952, were examined at intervals until March, 1953. As indicated in Figure 1, virus was present until the middle of November and was not detected during the winter months. On the basis of number of isolations from positive sewages, i.e., those containing virus (Figure 1), Coxsackie viruses were present in greatest amount from July to early September. Thereafter, the amount dropped off gradually.

Survival

In sewage treatment plant—In order to determine whether sewage treatment

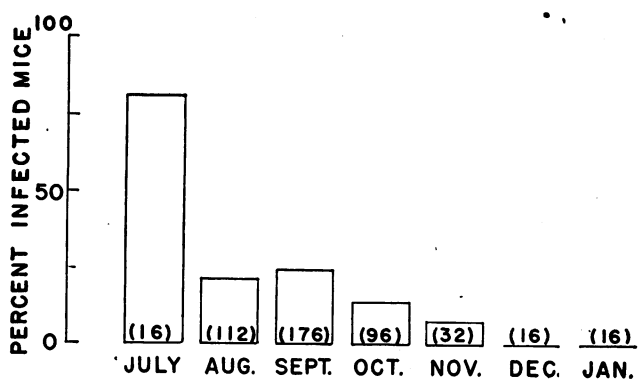

FIGURE 1—Frequency of Coxsackie virus isolation according to month. The figures in parentheses refer to the total number of animals inoculated.

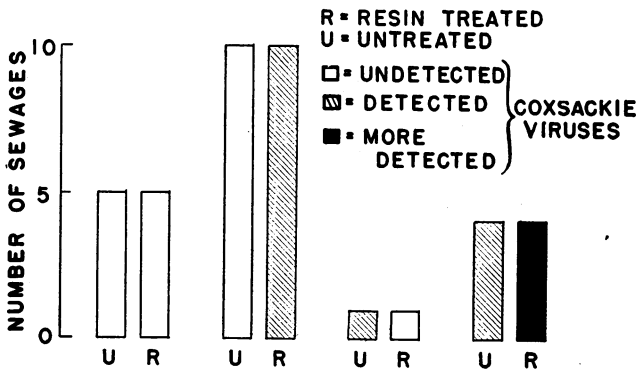

FIGURE 2—Comparison of sample treatment on frequency of Coxsackie virus recovery from sewage.

disposes of the Coxsackie viruses found in raw sewage, 48-hour swab samples from various stages in the Colonie sewage treatment plant were examined for virus (Table 7). In this plant sewage is subjected to the following treatment: primary sedimentation, trickling filter clarification, and secondary sedimentation with simultaneous chlorination. Viruses were detected only in the trickling filter dosing tank and in the trickling filter effluent. At the time of testing, none were detected in the raw sewage influent, the primary sedimentation tank influent, or the secondary sedimentation tank effluent.

TABLE 7

Coxsackie Viruses in Colonie Sewage Treatment Plant

Sample	*Dates of Collection*	
	10/14–16/52	*11/5–7/52*
Raw sewage influent	—	—
Trickling filter dosing tank	+	—
Trickling filter effluent	+	+
Plant effluent	—	—

In storage—For convenience, before testing for the presence of virus, sewage samples and swabs were frequently frozen at —55° C. In many laboratories such storage for preserving viruses in tissues or tissue suspensions is routine. This procedure did not reduce infectivity of the samples for at least a five-month period (Table 8).

TABLE 8

Coxsackie Virus Recovery by Resin Adsorption After Sewage Storage

Storage Interval	*No. Infected/16 Inoculated Animals*	
	Original Test	*Final Test*
8/21/52 — 11/ 7/52	3	3
9/10/52 — 11/ 7/52	12	11
10/16/52 — 3/13/53	2	2

Testing Method

That resin adsorption improves the detection of Coxsackie viruses in sewage is clear because (1) virus was detected in resin-treated samples and not in the same samples untreated; and (2) more virus was detected in resin-treated samples than in the same samples untreated shown to contain Coxsackie viruses. Of 15 samples not infective in the original state, virus was detected in 10 after the samples were resin treated. Of five samples containing detectable virus, a greater amount was evident in four following resin treatments (Figure 2). The viruses were detected with greater frequency, also, when samples were resin treated than when untreated, as indicated in Table 4. During the testing period involved, August–December, resin treatment of samples gave the most consistent yield of virus (Table 5).

Reproducibility

A necessary attribute of a detection method is its reproducibility. The results of the storage experiment described

above (Table 8), also indicate that resin treatment of sewage samples gives reproducible amounts of virus.

Types Isolated

Most of the strains isolated had the characteristics of Coxsackie Group A viruses; their serological classification will be discussed in a later report. To determine if resin treatment were limited in its capacity to the recovery of Coxsackie Group A virus from sewage, sewage samples were inoculated with known amounts of Group A (Type 10) and Group B (Type 1) strains, and their recovery by resin adsorption determined. As indicated in Table 9, Group B virus was recovered to the same extent as Group A.

TABLE 9

Recovery of Groups A and B Coxsackie Viruses from Resin

Fraction	Vol. (ml.)	M.E.D. (neg. log)	
		Group A	Group B
Original suspension	100	6.4	7.5
Eluate	2	7.9	8.5

DISCUSSION

The data given above suggest that Coxsackie viruses are found seasonally in sewage. This seasonal appearance may be due to (1) continuous discharge of virus into domestic sewage and its failure to survive winter conditions; and (2) seasonal discharge of virus into domestic sewage. Indeed, most of the Coxsackie virus isolations from sewage reported previously have been in the summer and early fall.[8, 9, 19] The latter explanation is the more probable since seasonal temperature fluctuations in sewage are small; in addition, the survival of other microorganisms in sewage, such as the coliform bacteria, is relatively constant throughout the year. Further evidence for this explanation is given by Huebner's report[20] that Coxsackie virus recoveries from fecal specimens taken before, during, and after summer illnesses were limited to the acute stage and the one- to two-month period following.

The recovery of Coxsackie viruses in the trickling filter effluent when not detected in the preceding stages of sewage treatment is less controversial when the nature of trickling filter structure and action is considered. The large area of the filter and its slime growths present opportunities for lodging virus particles over a period of time; the sample in the filter effluent may consist of material other than that of the influent, since sewage constituents undergoing change by contact with the filter growths may be retained longer than the period of liquid sewage passage through the filter. The oxidizing action of the filter on sewage may liberate virus bound to some masking compound or may destroy an inhibitor. It is of interest to note that the bacterial virus, *E. coli* B bacteriophage, is not inactivated by trickling filter activity while coliform organisms, on the other hand, are markedly reduced by its action.

Since resin adsorption permits the detection of viruses in samples in which they are not detected without such treatment, an obvious explanation of resin behavior under these circumstances is that of virus concentration. An alternative suggestion is that in the process virus particles are separated from inhibitory substances. The correct interpretation of the resin's mode of action will require further study.

The method described above has certain attributes which recommend it and which are increasingly apparent with use:

1. The manipulations are not involved and can be done rapidly and in a routine manner.

2. They are such that the possibility of laboratory infection is not great. While the resin technic does not eliminate the need for vigilance in handling potentially infectious material, the hazards involved are less than those accompanying other concentration pro-

cedures, such as centrifugation and precipitation of large volumes.

3. The materials and equipment required are relatively inexpensive and readily available.

4. Several samples can be tested at one time.

5. In combination with the sewage swab, routine sampling covering a wide area can be practiced.

SUMMARY

A simple, rapid, and comparatively safe method for detecting Coxsackie viruses in sewage is described, involving adsorption on and elution from ion-exchange resins. With this procedure, sewages were examined for their Coxsackie virus content in relation to source, seasonal fluctuation, types isolated, survival during sewage treatment, and storage behavior.

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