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Enhancement of Infectivity of Poliovirus RNA with Diethylaminoethyl-Dextran (DEAE-D)*, **

By

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Introduction

The infectious activity detectable in preparations of nucleic acids extracted from animal viruses represents only a fraction of the infectivity of the virus. The remainder of the biologically active material is lost either in the assay itself or during the extraction (1). We have observed that diethylaminoethyl-dextran (DEAE-D) could produce a 3 to 4-fold increase in the number of plaques appearing in an ordinary enumeration assay of intact poliovirus. The effect leading to the increased efficiency of plating with mature virus was apparently exerted early in the post-adsorption period (2). In seeking an explanation of this phenomenon we noticed the augmenting effect of DEAE-D on infectious RNA (3). This paper reports further observations on a simple method of assay for infectious RNA, developed with the use of DEAE-D, that appears to be 100 times more sensitive than conventional methods. Preliminary experiments on the mode of action of this polycation as an enhancing agent in assays and as a stabilizing agent in the extraction of RNA are also presented here.

Materials and Methods

Cell Cultures and Media. Primary Rhesus monkey kidney (MK) cells and the continuous line of MK cells, LLC-MK-2 (4), were grown in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum, penicillin (100 IU/ml.), streptomycin (100γ/ml.) and nystatin (25 γ/ml.).

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Viruses. Pools of the Mahoney and the CHAT type 1 strains of poliovirus were prepared by inoculation of monolayers of LLC-MK-2 cells with virus at a high multiplicity of infection. No calf serum was added to the maintenance medium (MEM).

Virus Plaque Assay. Monolayers of primary MK cells in 60 mm plastic Petri dishes (Falcon) were inoculated in duplicate with 0.2 ml. of diluted virus. After 60 minutes at 37° C the monolayers were overlaid with medium consisting of MEM, 0.055% purified bovine albumin, 2.5% fetal calf serum, 0.9% Noble's agar, and antibiotics. A second overlay containing neutral red (1:10,000) was added 2 days later, and plaques were counted 3 and 4 days after infection.

Extraction of RNA. Monolayers of LLC-MK-2 or primary MK cells were infected with CHAT or Mahoney virus at a multiplicity of $\geqslant 10$. The infected cells were harvested in a small volume at the time of appearance of early cytopathic changes. The infectious RNA was prepared directly from the infected cells by two cycles of extraction with cold phenol according to *Wecker*'s method (5). Sodium dodecyl sulfate (SDS), 0.5%, was used with phenol in some of the extractions (6).

The DEAE-D Method of Assay of Infectious RNA (3). Monolayers of primary MK cells were washed once with 5 ml. of 0.14 M phosphate-buffered saline, free of Ca^{++} and Mg^{++} (PBS^{-}), and containing antibiotics. The washing fluid was aspirated completely. The monolayers were inoculated with 0.2 ml. of mixed equal parts of solutions of RNA and DEAE-D (Diethylaminoethyldextran, Pharmacia; prepared from dextran, M. W. $= 2 \times 10^6$). Dilutions of the RNA and the DEAE-D were made in PBS^{-} without antibiotics. After 15 minutes at 23° C the plates were washed once with 5 ml. of Hanks' balanced salt solution (BSS) containing antibiotics. The plates were overlaid, and the procedure for virus plaque assay was followed.

Infectious RNA was also assayed with 1 M $MgSO_4$ in Tris buffer, pH 7.3, (5) and in 0.8 M sucrose (7) on primary MK cells.

Results

Enhancement of Virus Plaque Counts

When DEAE-D was added to the diluted virus inoculum used in an assay of poliovirus, the number of PFU that appeared 3 to 4 days after infection was increased more then 3-fold (Table 1). The increase in PFU was dependent on the concentration of DEAE-D. The doses of DEAE-D giving the maximum number of plaques were between 30 and 300 γ/ml. DEAE-D was not toxic to the cells at a concentration of 100 γ/ml. under the conditions outlined in Table 1. With 300 γ/ml. the plaques were small and irregular in outline and, when unbuffered 0.14 M NaCl was used, reduced in number. When PBS with antibiotics or Hanks' BSS with antibiotics were used as the diluents the increase in plaque number was not as high.

Enhancement of Infectious RNA Plaque Counts

Table 2 gives the results of assays of infectious Mahoney RNA of poliovirus under isotonic conditions with DEAE-D. With isotonic medium, no plaques appeared in the absence of DEAE-D even at lower dilutions

(10^{-2} and 10^{-3}) of the RNA. The optimal concentration of DEAE-D was approximately 1000 γ/ml., but as little as 30 γ/ml. exerted an effect. The plaques that formed with 10,000 γ/ml. of DEAE-D were small and ragged, apparently due to toxicity of the DEAE-D.

When the same Mahoney RNA preparation was assayed by the most efficient conventional method (5) (in 1 M $MgSO_4$), no plaques appeared

Table 1. Effect of DEAE-D in the assay of intact poliovirus

DEAE-D in inoculum (γ/ml.)	PBS (0.14M)		NaCl (0.14 M)	
	(No. of PFU)	(% of Control)	(No. of PFU)	(% of Control)
300	75	326	24	120
100	63	274	56	280
30	58	252	63	315
10	35	152	47	235
0	23	100	20	100

Equal parts of DEAE-D and CHAT virus in the diluents indicated were mixed in tubes. Monolayers of primary MK cells were inoculated in duplicate with 1 ml. of the mixture and incubated at 37° C for 120 minutes before removal of the inoculum and overlaying.

Table 2. Assays of infectious RNA with DEAE-D in isotonic buffered saline (PBS$^-$)

DEAE-D (γ/ml.)	Polio RNA assays (PFU/0.1 ml.)			
	CHAT		Mahoney	
	(10^{-4})*	(10^{-5})	(10^{-4})*	(10^{-5})
10,000	10	2	10	N.D.**
3,000	16	0	29	N.D.
1,000	36	1	42	4
300	33	4	30	3
100	24	1.5	16	1
30	2	0	2	0
10	0	0	0	0
0	0	0	0	0

* Dilution of ethanol-precipitated RNA-containing material.
** Not done.

at the 10^{-4}, and there were only 2 plaques at the 10^{-3}, dilution. This contrasts with the 42 plaques observed at the 10^{-4} dilution when assayed with the DEAE-D method.

DEAE-D had a similar effect in an assay of infectious RNA in which hypertonic sucrose (final concentration, 0.8 M) was used instead of (PBS$^-$). There was, however, no additive effect with the hypertonic sucrose; in fact, the assay was slightly more sensitive when the isotonic (PBS$^-$) was used. DEAE-D did not exert an augmenting effect if used in conjunction with hypertonic $MgSO_4$ (3).

The infectiousness of the Mahoney RNA was destroyed by RNase and unaffected by DNase and diluted type-specific antiserum (Table 3). According to these criteria the infectivity of the extracted preparation was due to RNA.

Table 3. Characterization of RNA preparation

Treatment of RNA*	RNA assays (PFU/0.1 ml.)	
	DEAE-D (γ/ml): 1000	300
RNase (1 γ/ml.)	0	0
(0.1 γ/ml.)	0	0
DNase (10 γ/ml.)	18	14
Type 1 Antiserum**	28	20
None (Control)	27	18

* 10^{-4} dilution of Mahoney RNA; reactants incubated at 23° C for 30 minutes in PBS.
** A 10^{-5} dilution of Mahoney virus yielded an average of 0.5 PFU after incubation at 37° C with the same dilution of antiserum. (30 PFU observed after incubation in PBS without antibody.)

Conditions of Adsorption

The usual conditions of adsorption in the DEAE-D method are 15 minutes at 23° C, followed by a single washing (3). A longer period of adsorption and both lower and higher temperatures failed to improve the sensitivity of the assay. A 5-minute period of adsorption seemed too brief (Table 4).

If the infected monolayers were washed more thoroughly at the end of the adsorption period, the sensitivity of the assay was reduced (Table 5). The reduction in the number of PFU brought about by the additional washing could be due either to removal of RNA or of DEAE-D. There is indirect evidence to support the latter possibility (see below).

Table 4. Effect of conditions of Adsorption in DEAE-D method of assay of infectious RNA

Temperature	Period before washing inoculated monolayers					
	5 Min.		15 Min.		60 Min.	
	(1000)*	(300)*	(1000)	(300)	(1000)	(300)
4°	—	—	9.5**	12	—	—
23°	3	2.5	9	7	10	11.5
37°	—	—	14	10	—	—

* Amount of DEAE-D (γ/ml.) used in assay.
** Number of PFU/0.1 ml. at $10^{-4.3}$ (CHAT RNA).

Treatment of MK Cells with DEAE-D and Other Substances before Assay of RNA with DEAE-D

Experiments were performed in an attempt to increase the sensitivity of the assay and to elucidate the mode of action of the DEAE-D. One ml. of the substances listed in Table 6 dissolved in BSS was added to mono-

layers of MK cells; the plates were placed at 37° C for 2 hours. The solutions were thoroughly aspirated from the monolayers, but only the monolayers pretreated with DEAE-D were washed before inoculation. The monolayers were then inoculated with RNA both with and without DEAE-D in (PBS$^-$) after 15 minutes at 23° C, and the monolayers were washed once and overlaid.

Table 5. Assays of infectious RNA with DEAE-D: effect of number of post-inoculation washings

DEAE-D (γ/ml.)	Number of washings 15 minutes after inoculation	
	1	3
10,000	10*	8
3,000	16	4
1,000	36	20
300	33	14
100	24	3
30	2	1
10	0	0
0	0	0

* Number of PFU/0.1 ml. at 10^{-4} (CHAT RNA).

Table 6. Effect of various kinds of pretreatment of the cell monolayers on the DEAE-D assay

Pretreatment of MK monolayers	RNA assays			
	DEAE-D (γ/ml.): 1000 (10^{-4})	300 (10^{-4})	0 (10^{-3})	0 (10^{-4})
None (Hanks BSS)	>70	58	0	0
DEAE-D (1000 γ/ml.)*	28	27	22	2
131 A (100 γ/ml.)**	20	4.5	0	0
DMSO (10%)	6†	8†	0†	0†

* Monolayers washed 3 times before inoculation with RNA.
** A polyanion containing carboxyl groups (8).
† DEAE-D and RNA diluted in 10% DMSO in PBS$^-$.

Cell cultures pretreated with DEAE-D were sensitive to infection with RNA: 22 PFU were observed after inoculation of the monolayers with a 10^{-3} dilution of RNA unmixed with DEAE-D. This occurred under isotonic conditions. Pretreatment with DEAE-D thus gave a definite effect which was similar to but less than that produced by the simultaneous addition of DEAE-D and RNA.

No plaques formed at dilutions of 10^{-3} or 10^{-4} with any of the other substances unless DEAE-D was used in the assay (Table 6). The substances included Compound 131A (Leo, Hälsingborg, Sweden), a strongly anionic soluble polymer (8), and dimethyl sulfoxide (DMSO). Pretreatment with

these substances failed to enhance the sensitivity of the DEAE-D assay in the usual isotonic medium. Pretreatment of the assay cells with either sodium dextran sulfate 2000 (Pharmacia) or hyaluronidase Type 1 (Sigma) followed by a DEAE-D assay of RNA also did not enhance the sensitivity. Neither did pretreatment of monolayers with DEAE-D followed by hypertonic $MgSO_4$ assay increase the plaque counts.

Extraction of Infectious RNA with DEAE-D Present

It was possible that most of the undegraded RNA present in the preparation at the time of assay had, in fact, been detected as infectious units with the DEAE-D method. It was also possible that DEAE-D introduced during the extraction process might function as a stabilizing agent. Accordingly we attempted to improve the yield of RNA from the virus preparations.

Table 7. Extraction of infectious RNA in the presence of DEAE-D

Concentration of DEAE-D in extraction (γ/ml.)	Method of extraction*	
	Cold phenol	Phenol and sodium dodecyl sulfate
3000	$10^{4.9}$**	—
300	$10^{4.9}$	$10^{7.0}$
30	$10^{6.4}$	$10^{6.0}$
0	$10^{6.5}$	$10^{6.5}$

* Titer of Mahoney virus before extraction, $10^{8.3}$ PFU/ml.

** Number of PFU/ml. in the ethanol-precipitated RNA- containing material (Mahoney); assay by DEAE-D method (300 γ/ml.).

In the first experiment 30,000 γ/ml. of DEAE-D added during phenol extraction of CHAT virus produced a visible precipitate in the RNA-containing phase, and no infectious RNA was recovered. However, 3000 and 300 γ/ml. of DEAE-D reduced the titer of the biologically active RNA only slightly.

The extractions were repeated with a range of concentrations of DEAE-D, and both with and without 0.5% SDS (Table 7). A volume of 4 ml. of Mahoney-infected MK cells, allotted to each combination of DEAE-D and SDS, yielded 0.04 to 0.05 ml. of RNA-containing material after washing. The highest absolute titer — $10^{7.0}$ PFU/ml. — was obtained with a combination of DEAE-D and SDS. If SDS was not used, the DEAE-D again apparently interfered with the recovery of infectious RNA (Table 7). In the extraction with SDS and 300 γ/ml. of DEAE-D, a total of $10^{5.6}$ PFU were detected in the RNA prepared from $4 \times 10^{8.3}$ PFU of virus.

Discussion

The mechanism of enhancement of both the intact virus and the infectious RNA demonstrated in these experiments may be linked in some way. Even without an understanding of the action of DEAE-D, the phenomenon will be quite useful. In the case of mature virus, it may allow a significant reduction in the high ratio of noninfectious to infectious particles existent in preparations of poliovirus. In the case of infectious RNA, the sensitivity of the DEAE-D assay may permit productive experiments with labeled RNA.

Effect on Virus. The effect of DEAE-D is apparently not due to the dispersal of clumped virions or to the facilitation of adsorption. DEAE-D must, however, be present at some time during the first 3 hours after infection for a clear-cut effect (2). Reversal of the action usually attributed to the inhibitors contained in agar is not responsible for the increased number of plaques (2). The effect of DEAE-D may be to increase the proportion of virus particles that complete the replicative cycle in the cell and may be associated in this way with the effect on RNA.

A practical point is that the commonly used antibiotics can interfere with the effect of DEAE-D. Streptomycin is particularly suspect because of its ionic binding ability. Considerations such as this suggest that the optimal virus-enhancing effect with DEAE-D has not yet been obtained.

Effect on Infectious RNA. The enhancement of assayable infectious RNA could be due to the formation of a stabilizing coating on the RNA molecules by DEAE-D. The occurrence of a direct, irreversible effect can be tested by exposure of RNA in high titer to DEAE-D followed by a dilution of the mixture sufficient to reduce the DEAE-D to an ineffective level. In a preliminary experiment treatment of RNA with DEAE-D prevented the destruction of its infectiousness by RNase (2). Even if DEAE-D does combine with the RNA and act as a stabilizer, an effect on the assay cells may still be essential. In contrast the somewhat similar though weaker enhancing effect produced by histones on infectious RNA is exerted by a direct action on the RNA and is apparently not mediated by an action on the assay cells (9).

DEAE-D may cause a change in the assay cells that facilitates the entrance of RNA. Some kind of cellular effect is apparently involved in the conventional methods of assay with hypertonic salts or sucrose. It should be emphasized that the molar concentrations of the solutions used in the DEAE-D method of assay are negligible. One possibility is that an alteration of the surface charge of the assay cells by the polycationic DEAE-D is involved in the action. The results of pretreatment of assay cells with DEAE-D and the effect of repeated washing following the usual DEAE-D method of assay point to some kind of cellular effect. Some

DEAE-D, however, may be available through leakage at the cell surface for interaction with RNA, or the effect may not be readily reversible.

An action on both the RNA and the cell may be involved. The cationic DEAE-D might counteract the repulsion between the negatively charged RNA and the negatively charged cell surface by forming complexes with the RNA that are attracted to the assay cells.

Extraction of Infectious Nucleic Acids. There is now reason to expect that an amount approaching 1% of the RNA can be recovered in an infectious form from poliovirus. Experiments have been performed based on the possibility that DEAE-D could function as a stabilizer during the extraction of RNA. It has yet to be shown that DEAE-D in the extraction gives an actual increase in the amount of recoverable RNA. However, the results are promising when both DEAE-D and SDS are added to a phenol extraction.

During these studies we observed that a precipitate forms when nucleic acids and DEAE-D are present in high concentrations. Extractions from purified virus instead of from infected cells may result in preparations of infectious RNA of sufficient titer with which to conduct high multiplicity of infection experiments. On the other hand, such a precipitating effect of DEAE-D on RNA might be put to use in a method of extraction.

Other Applications. There are several areas for further investigation. The DEAE-D technique might be extended to the assay of the infectious RNA of other animal viruses. DEAE-D might be applied to the assay of bacteriophage RNA — a theoretically interesting possibility because of the different nature of the host cells. A more sensitive system of assay for infectious DNA, especially from a tumor virus such as SV_{40}, is highly desirable because of the high ratio between infectious units and cell-transforming units. DEAE-D might make possible the recovery in an infectious form of the nucleic acid from a virus when this has hitherto failed because of instability of its nucleic acid or lack of a suitable system of assay.

Summary

Diethylaminoethyl-dextran (DEAE-D) greatly increases the detection of infectious RNA in MK cells. The DEAE-D is the basis of a simple method of plaque assay for poliovirus RNA under isotonic conditions that is 100 times more sensitive than conventional methods. The same polycationic substance yields a 3 to 4-fold increase in plaque counts when used in the assay of intact poliovirus.

Temperature and duration of adsorption have little influence on the DEAE-D method of assay of RNA. A somewhat lesser, but definite, enhancing effect is obtained when the assay cells are exposed to DEAE-D and washed repeatedly before inoculation with RNA.

The presence of DEAE-D in extractions of poliovirus RNA with cold phenol and sodium dodecyl sulfate may improve the yield of infectious RNA. A total of $10^{5.6}$ PFU of Mahoney RNA were detected in the extract made directly from 4 ml. of infected MK cells with a titer of $10^{8.3}$ PFU/ml. The absolute titer in the ethanol precipitate was 10^7 PFU of RNA per ml. determined with the DEAE-D method.

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