Enhancement of Polio-RNA Infectivity by Dimethylsulfoxide. (31508)

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Nucleic acid extracted from certain viruses is infectious *in vitro* and *in vivo,* but represents only a small fraction of the infectivity of the whole virus(1). Several methods have been shown to enhance the infectivity of the viral nucleic acid; these include the use of hypertonicity(2), histone (3), protamine(4) and DEAE-dextran(5,6). Because the level of infection produced by nucleic acid even in the presence of these enhancing agents is only about 0.1 to 0.5% of the viral infectivity, the possibility of producing infection by nucleic acids from viruses of characteristically low infectivity is greatly reduced. It therefore would be useful to continue a search for methods to enhance nucleic acid infection and to elucidate the mechanism of such enhancement.

Kligman(7) has recently reviewed the clinical and experimental results obtained with the use of dimethylsulfoxide (DMSO) which is found to enhance the absorption of a wide variety of compounds. The absorption-enhancing properties of DMSO suggested that it might be useful to increase the infectivity of viral nucleic acid. A preliminary report from this laboratory indicated that enhancement of nucleic acid infection with DMSO was possible(8). Dimethylsulfoxide was tested for its ability to enhance cellular infection by polio-ribonucleic acid (RNA). The experiments reported in detail here demonstrate that DMSO enhances polio-RNA infectivity for primary African green monkey kidney cell monolayers. These experiments further suggest that DMSO is superior to other enhancing agents compared under the same conditions in this test system.

Materials and methods. Primary African green monkey kidney (GMK), primary rabbit kidney (RK) and HeLa cell cultures were obtained from the Tissue Culture Section, Division of Biologics Standards, National Institutes of Health and maintained in Eagle's Basal Medium containing 2% chick serum, M-199 containing 2% fetal bovine serum and Eagle's Basal Medium containing 10% calf serum, respectively.

A high passage poliovirus Type 1, Mahoney strain, grown in Hep-2 cells was used in all experiments.

Extraction of polio-RNA. The procedure used in these experiments was a modification of that used to extract polyoma viral nucleic acid(9). Poliovirus at a concentration of 6.5 to 7.5 $\log_{10}$ $TCD_{50}/1.0$ ml was inoculated into 32 oz culture bottles containing approximately 4×10^7 HeLa cells. The cells were harvested 8-10 hours after inoculation by scraping with a rubber policeman, collected by low speed centrifugation and suspended in 10 ml each of 0.04% versene, saline-citrate (0.015 *M* sodium citrate in 0.14 *M* NaCl), 0.15 *M* trichloracetic acid (titrated to pH 7.4 with *M* KOH) and 5 g of 90-mesh Alundum. The suspension was stirred vigorously with a magnetic stirrer for 30 minutes at 4°C. An equal volume of phenol, saturated with 0.01 *M* phosphate-buffered saline, pH 7.4 (PBS) was added and the suspension shaken vigorously by hand for 8 minutes, followed by centrifugation at 2,000 rpm for 40 minutes at 4°C. The aqueous and interphases were re-extracted with saturated phenol and separated by centrifugation as before. The aqueous phase was removed and the nucleic acid precipitated by addition of 4 volumes of absolute alcohol. After 2 hours at 4°C, the flocculent nucleic acid was collected by centrifugation at 2,000 rpm for 30 minutes, washed once with absolute alcohol and dissolved in 4 ml of PBS. The nucleic acid solution was then extracted 3 times with anhydrous ether and the excess ether blown off under nitrogen or air.

Infectivity assays. Aliquots of the nucleic acid were initially diluted 1:10 in varying concentrations of DMSO made up in PBS. The same dilution was also made in *M* $MgSO_4$, in 0.01 *M* Tris buffer, pH 7.8 and in PBS alone or with protamine (40 μg/ml), histone (400 μg/ml) or DEAE-dextran (500 μg/ml) added. Further dilutions in 5-fold or 10-fold steps were made in these diluents. Monolayers of GMK or RK cells in stationary test tube cultures, from which the medium had been removed, were inoculated with 0.1 ml of nucleic acid solution in the appropriate diluent and incubated at room temperature for 20 minutes. With DMSO, absorption time and temperature were varied from 2 to 30 minutes and from 2°C to 37°C, respectively. The lowest dilution of nucleic acid was also mixed with an equal volume of type-specific polio-immune horse serum (diluted to neutralize 1,000 TCD_{50} of poliovirus per 0.1 ml of serum) or with 100 μg/ml of pancreatic ribonuclease (RNAse). After incubating for one hour at room temperature the serum and RNAse-treated nucleic acid solutions were inoculated as described above.

Following the absorption period the cell sheets were washed twice with 2 ml of maintenance medium, fed 1 ml of medium and incubated at 35°C. Five tubes per dilution were used; these were observed daily for the appearance of cytopathic effect (CPE) and the titer calculated by the Karber method (10) for 50% tissue culture infectious dose (TCD_{50}) and expressed per milligram of RNA.

Intact poliovirus was also titrated using 20% and 40% DMSO or 500 μg/ml DEAE-dextran in PBS as diluents. The RNA content of the preparation ranged from 200-800 μg/ml as estimated by the orcinol reaction (11). Deoxyribonucleic acid was estimated by the method of Webb and Levy(12) and found to be <10 μg/ml in most preparations of infectious polio-RNA. The method of Lowry *et al*(13) was used for protein determinations; all RNA preparations contained <50 μg/ml protein.

Results. Typical enteroviral CPE was produced by all RNA preparations from polio-infected HeLa cells. This infection was not inhibited by immune serum or by 100 μg/ml of deoxyribonuclease but it was completely abolished by RNAse (50 μg/ml). Infection of RK cells with polio-RNA was demonstrated according to Holland *et al*(14).

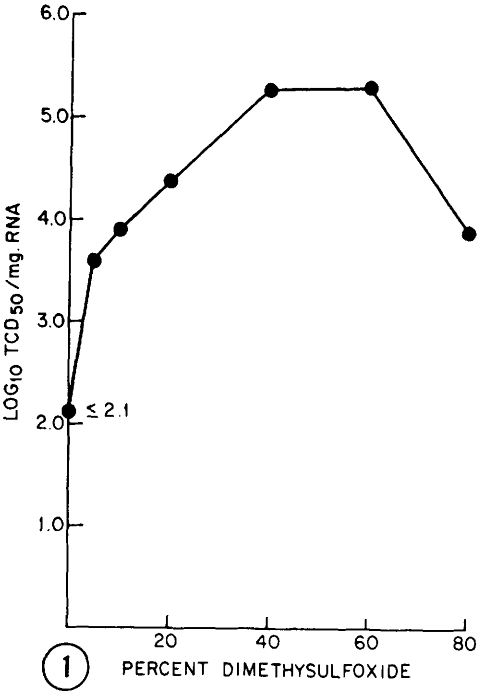
A problem in using DMSO was its pronounced toxicity for cultured cells when incubated with them over prolonged periods. This toxic effect was observed when concentrations of DMSO greater than 3% were incubated with GMK cells for periods of several hours or longer. In order to circumvent DMSO toxicity, a small volume (0.1 ml) of nucleic acid-DMSO solution was inoculated onto cell monolayers for less than 30 minutes followed by 2 or more washes with culture medium. This procedure prevented cytotoxic effects up to 60% DMSO with cultures remaining in good morphologic condition up to 14 days.

Using this procedure the usual increase in infectious titer from RNA preparations alone to that obtained with DMSO was 3 $\log_{10}$ TCD_{50}/mg RNA. Fig. 1 shows that DMSO enhanced the RNA infection with a nearly linear increase in polio-RNA titer resulting when DMSO was increased from a concentration of 5% to 40%. The optimal DMSO concentration for enhancement was 40% when using a 20-minute absorption period at room temparature. The decrease in titer with 80% DMSO is probably due to marked cytotoxicity, making CPE impossible to read at 5 days after inoculation.

Absorption of the RNA solution was investigated by varying the time that the RNA-DMSO inoculum was in contact with GMK cells. Fig. 2 illustrates that a significant fraction of infectious RNA was absorbed within the first 2 minutes. The infectivity then appears to plateau at 20 minutes with no further increase in titer noted between 20 and 30 minutes. The increase in titer between 2 and 20 minutes was 0.8 $\log_{10}TCD_{50}$/mg RNA.

The effect of temperature on DMSO-enhanced polio-RNA infection was studied by varying the temperature at which absorption occurs. Absorption was carried out in an ice bath (2°C) at room temperature (22°C) and in 31°C and 37°C incubators. The data shown in Fig. 3 indicated that absorption carried out at 22°C resulted in significantly greater infectivity titers than the other temperatures tested.

The enhancing ability of 40% DMSO for infectious polio-RNA was compared to several other methods of enhancement. Serial dilutions of polio-RNA were made in 40% DMSO, *M* MgSO$_4$, protamine, histone, and DEAE-dextran. All solutions were prepared



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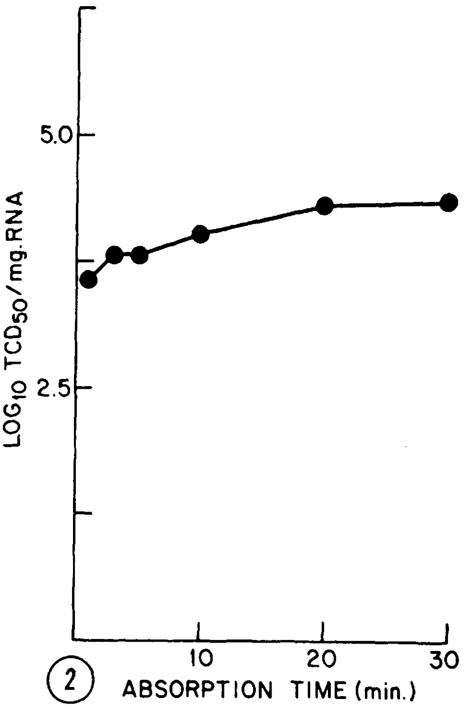

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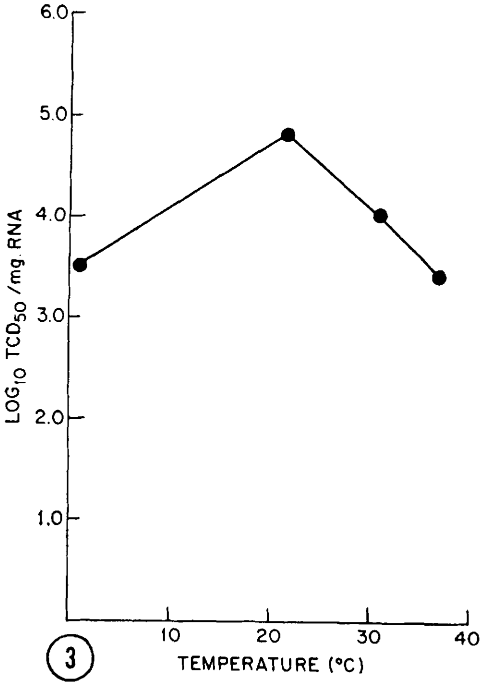

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FIG. 1. Effect of varying the concentration of DMSO on titer of infectious polio-RNA. Serial 10-fold dilutions of RNA from polio-infected HeLa cells were made in 5-80% DMSO. Replicate titrations of infectious nucleic acid were made in GMK cells and titers plotted against increasing DMSO concentrations; absorption time was 20 min at room temperature.

FIG. 2. Effect of varying absorption time on the titer of infectious polio-RNA. Nucleic acid was titrated in the presence of 40% DMSO at room temperature.

FIG. 3. Effect of varying temperature on absorption of infectious polio-RNA in 40% DMSO; absorption time at all temperatures was 20 min.

TABLE I. A Comparison of Agents Enhancing Polio-RNA Infection in GMK* Cultures.

	RNA preparation No.				
Agent	1	2	3	4	5
Buffered saline	≤1.5†	≤ .3	≤2.1		2.1
M $MgSO_4$	2.8	1.8	2.8		2.9
Histone (400 μg/ml)	2.0	2.3	2.8		
Protamine (40 μg/ml)	3.3	4.4	4.0 3.7		3.5
DEAE-dextran (500 μg/ml)				4.0	4.1
40% dimethyl sulfoxide			5.3 5.1	4.8	4.5

* Primary African Green Monkey.

† Titers expressed as $\log_{10}$ TCD_{50}/mg RNA.

in PBS except $MgSO_4$, which was prepared in 0.01 M Tris buffer, pH 7.8. Replicate titrations of polio-RNA were then made in GMK cells using an inoculum of 0.1 ml and incubating the cultures for 20 minutes at room temperature.

Comparison of 5 RNA preparations in several experiments is presented in Table I. All agents enhanced polio-RNA infection, as indicated in the Table; however, enhancement was greatest using 40% DMSO. For all RNA preparations in which M $MgSO_4$ and histone were compared, no difference in polio-RNA titer was noted. Protamine, on the other hand, was found to produce consistently higher infectious titers than either M $MgSO_4$ or histone. DEAE-dextran appeared to produce slightly higher titers than did protamine. The average titer of polio-RNA infectivity obtained with DMSO-enhancement was 4.9 $\log_{10}$ TCD_{50}/mg RNA compared with a titer of 4.1 $\log_{10}$ for DEAE-dextran-enhancement.

The effect of DMSO and DEAE-dextran on the infectivity titer of poliovirus was studied. Ten-fold serial dilutions of virus in 20% and 40% DMSO and in 500 μg/ml DEAE-dextran were made and 0.1 ml was inoculated into each of 5 GMK cultures per dilution. Data from these experiments are presented in Table II. It is apparent that there was neither inhibition nor enhancement of intact virus infectivity.

The infectivity achieved with polio-RNA enhanced with DMSO was 0.5 to 0.8% of the virus infectivity. This is a significant increase over nucleic acid infection obtained without the use of an enhancing agent.

Discussion. The above results indicate that DMSO appears to be useful for enhancing polio-RNA infection. The mechanism of action for this enhancement is unknown, as is the case in DMSO-enhanced absorption of many compounds(7). However, certain facts concerning DMSO are known. It has been shown that DMSO does not damage intact sheets of stratus corneum(7) or does it appear to damage cell membranes at concentrations of 10% as seen by the electron microscope (15). Further, there is no loss of viral ultrastructure when cells are frozen and thawed in 10% DMSO(16).

The possibility that DMSO as used in these experiments may be exerting its enhancing effect by osmotic shock (hypertonicity) is necessarily considered. However, the polio-RNA infectivity achieved with all concentrations of DMSO is greater than that resulting from M $MgSO_4$. It has been shown that increasing concentrations of salt above 1.0 M do not increase infectivity(2). Although the osmotic effect of DMSO can not be discounted, there may be an added mechanism of action to achieve infectious titers 100-1,000-fold greater than those obtained with M $MgSO_4$.

DMSO does not protect RNA from RNAse since this enzyme destroys the biological activity of polio-RNA as noted in these experiments. In addition, when the RNA preparations are diluted in 40% DMSO, RNAse reduced the amount of nucleic acid precipitated by 5% trichloracetic acid to the same extent that it did in a saline solution(17).

TABLE II. Effect of Two Enhancing Agents on Poliovirus Titers.*

	Titer	
Agent	Exp 1	Exp 2
None	6.1	6.1
20% DMSO	5.9	
40% DMSO	5.7	
DEAE-dextran (500 μg/ml)		6.1

* Expressed as $\log_{10}$ TCD_{50}/0.1 ml; serial 10-fold dilutions were made in each agent in PBS.

The kinetics of infectious polio-RNA absorption in the presence of 40% DMSO indicate that absorption occurs promptly with a peak achieved at 20 minutes. This agrees with data from histone stimulated enhancement of infectious nucleic acid(3) and also agrees with the kinetic data of Ellem and Colter(18) who used hypertonic sucrose. The data of Pagano and Vaheri(5) using DEAE-dextran to enhance infectious RNA uptake also indicate a maximum absorption period of 15-20 minutes.

As noted, there was no enhancement of intact virus infectivity with either DMSO or DEAE-dextran. This contrasts with a report of enhanced virus infectivity resulting from the use of DEAE-dextran(5). This lack of agreement may be explained on the basis that the present experiments were carried out using a 50% tissue culture infectious dose titration, whereas a plaque titration was used in the earlier report. It is also possible that DEAE-dextran is acting to bind or reduce inhibitors known to be present in agar (19), consequently increasing the efficiency of plaque formation.

Dimethylsulfoxide is a useful agent for increasing the sensitivity of assaying infectious polio-RNA and presumably, all infectious nucleic acids. Although there are many factors which may preclude the isolation of an infectious nucleic acid, DMSO may make it possible to assay the infectivity of nucleic acids from some viruses of characteristically low infectivity titers.

Summary. Dimethylsulfoxide (DMSO) has been shown to enhance infection by polio-RNA. This enhancement was present for all concentrations of DMSO from 5% to 80%. The enhancement was optimal at 40% DMSO with a 20-minute absorption period at room temperature. A significant percentage of the nucleic acid infectivity was absorbed in the first two minutes. Dimethylsulfoxide enhanced RNA infection to a greater degree than *M* $MgSO_4$, protamine, histone, and DEAE-dextran when tested under the same conditions. There was no enhancement of intact viral infectivity by either DMSO or DEAE-dextran.

ADDENDUM: Recently Koch *et al*(20) have shown an enhancement of polio-RNA infectivity using Poly-L-Ornithine which appears to be slightly greater than DEAE-dextran.

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