in establishing cross tolerance against $G_{60}A_{40}$. Neither polyglutamic acid nor $G_{60}L_{40}$ when injected neonatally, were effective in reducing an adult response against $G_{42}L_{28}A_{30}$. However, $G_{60}A_{40}$ did reduce the response against a first course of immunization with $G_{42}L_{28}A_{30}$. This tolerance was broken by a second immunization with polymer in complete adjuvants.

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Inhibition of Poliovirus Minute-Plaque Mutants and Echoviruses by Sulfated Polysaccharides.* (30059)

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Takemoto and Liebhaber(1) reported that dextran sulfate inhibited multiplication of encephalomyocarditis (EMC), echo-, coxsackie-A9 and herpes viruses and enhanced plaque-forming capacity of poliovirus type 1 Mahoney. Sulfated polysaccharide in agar has been shown to inhibit multiplication of herpes virus(2), dengue virus(3), EMC virus (4), and a *minute-plaque* (*m*) mutant of poliovirus(5).

The effects of natural and synthetic sulfated polysaccharides on growth of 4 *m* mutants of poliovirus and on plaque formation by these and certain prototype echo- and wild-type polioviruses are reported herein.

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A comparison of the effects of L-cystine and diethylaminoethyl (DEAE)-dextran on plaque diameter and efficiency of plating (e.o.p.) of polio- and echoviruses is also made.

Materials and methods. Cells. Primary rhesus monkey (*Macaca mulatta*) kidney cells were grown in Petri plates on high-cystine altered Eagle's maintenance ($hcAE_m$) medium(6) containing 4% calf serum, as previously described(7).

Viruses. Three *m* mutants of type 1 Brunhilde poliovirus, designated m_{alpha}, m_{pi}, and $m_{epsilon}$, were available; Chapin and Dubes(8) isolated the mutants by passaging wild-type Brunhilde (propagated and plaque-purified on monolayers of primary monkey kidney) on established monkey kidney lines through 10 serial passages in liquid medium. Stocks of wild-type poliovirus strains Akron (type 1) and Mahoney (type 1) and its mutant, slow Mahoney (obtained by passaging wild-type Mahoney on monolayers of HeLa cells)(9), wild-type strains Charbonneau (type 2), Theiler's Lansing (type 2), and Mabie (type 3) and prototype viruses of ECHO 5 (strain Noyce), ECHO 8 (strain Bryson), ECHO 11 (strain Gregory), and ECHO 26 (strain Coronel 11-3-6) were available in this laboratory(10).

Agar extract. Agar extract was prepared by mixing 1 volume of 2.7% melted agar with 2 volumes of 3× concentrated $hcAE_m$ medium. The mixture was then frozen at −20° and thawed. The supernatant was filtered through gauze and used as a source of agar extract.

DEAE-dextran and sodium dextran sulfate. DEAE-dextran and sodium dextran sulfate were obtained from Pharmacia, Uppsala, Sweden. Stock solution of dextran sulfate (DS) was prepared by dissolving appropriate concentration of sodium dextran sulfate to contain 4,000 μg/ml and dispensing into small containers. DEAE-dextran stock solution was similarly prepared in boiling water.

Plaque assay. Monolayers of primary monkey kidney cells were washed twice, each time with 4 ml of phosphate-buffered saline (PBS)(11), then inoculated with appropriate concentrations of virus. Following one hour adsorption at 37° plates were overlayered with 4-6 ml of $hcAE_m$ containing 0.9% agar. DEAE-dextran and DS were used in concentrations of 100 μg/ml, unless otherwise specified in the results. Plates were incubated at 36-37° in the humidified atmosphere of a continuous-flow incubator flushed with a mixture of 3% CO_2 and 97% air. Following 3-5 days plaques were revealed by addition of a second agar overlay containing 0.01% neutral red and plaques were scored.

Tests of cytopathic effect (CPE). Tubes containing monolayers of primary monkey kidney cells were washed twice, each time with 4 ml PBS, then inoculated with 0.1 ml of appropriate virus concentration. Following 1 hour adsorption at 37°, 1 ml of $hcAE_m$ was added to each tube. In tests for inhibition of viral CPE 100 μg/ml (unless otherwise specified) of DS were incorporated into the medium. Cultures were placed on a rotating drum; they were observed under the microscope for CPE.

Results. Effect of various concentrations of DEAE-dextran on plaque diameter. Takemoto and Liebhaber(4) have shown that a sulfated polysaccharide present in agar inhibits plaque formation by wild-type EMC virus. Addition of DEAE-dextran to the agar overlay abolishes this inhibition by binding the sulfated polysaccharide and results in greatly increased plaque diameters(12). Experiments were conducted to test possible inhibition by agar of 4 *m* mutants of poliovirus. Fig. 1 shows a comparison of plaque sizes of Brunhilde wild-type, its 3 *m* mutants and Mahoney wild-type (fast) and its slow (also *m*) mutant with and without DEAE-dextran in the agar overlay. Plaque diameters of all 4 mutants tested were greatly increased by addition of 100 μg/ml DEAE-dextran to the agar overlay. It should be noted that in Fig. 1 concentrations of virus stocks in inocula were chosen to illustrate plaque sizes; thus 2 plates of any pair have not necessarily been inoculated with the same virus concentration.

In other experiments we noted that certain echoviruses form minute plaques (0.8-2.0 mm in diameter) following 5 days' plaque development under agar. Echoviruses 5, 8, 11, and 26 were included with poliovirus *m*

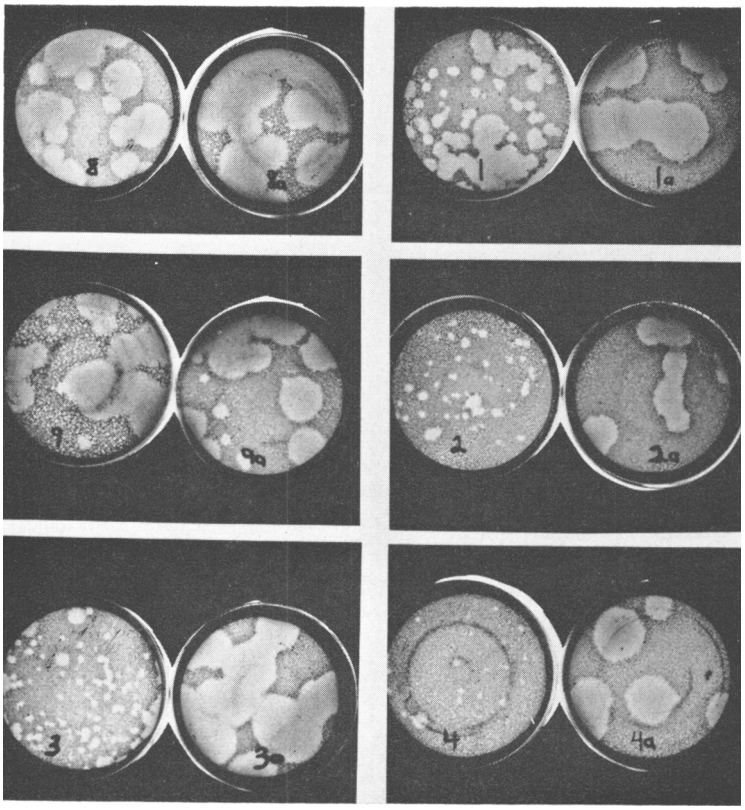

FIG. 1. Comparison of plaque sizes of wild-type poliovirus strains Mahoney(8) and Brunhilde(9), slow Mahoney mutant (1) and Brunhilde minute-plaque mutants m_{alpha} (2), m_{pi} (3), and $m_{epsilon}$ (4) on standard agar-hcAE$_m$ medium with those on agar-hcAE$_m$ + 100 μg/ml DEAE-dextran (designated by corresponding numbers followed by "a").

mutants in studies on the relationship between concentration of DEAE-dextran in the overlay and plaque diameter. Results in Table I show that plaque diameter of each of the poliovirus mutants and echovirus mutants and echoviruses tested was increased with increased concentrations of DEAE-dextran up to 200 μg/ml. Additional experiments indicated that any further concentration of DEAE-dextran up to 1,000 μg/ml gave essentially the same results as 200 μg/ml.

Effect of DEAE-dextran on plaque diameter and e.o.p. as a function of plaque develop-

TABLE I. Effect of Various Concentrations of DEAE-Dextran on Plaque Diameters of Poliovirus Mutants and Echoviruses.

Virus	Mean plaque diameter (mm)* Concentration of DEAE-dextran in overlay (μg/ml) 0	12.5	25	50	100	200
Brunhilde mutant m_α	1.0	1.0	2.0	5.0	5.0	9.0
" " m_π	1.0	1.0	3.5	4.0	4.0	10.0
" " m_ϵ	1.0	1.5	2.0	4.0	6.0	12.0
Slow Mahoney mutant	2.0	2.0	3.0	7.5	12.0	12.0
ECHO 5, wild	1.0	1.0	2.0	4.0	8.0	10.0
" 8, "	1.0	1.0	2.5	4.0	8.0	12.0
" 11, "	1.0	1.0	2.0	3.5	8.5	12.0
" 26, "	1.5	1.5	2.5	4.0	8.0	12.0

* Mean plaque diameter based on an average of 20 well-separated plaques from 5 plates.

TABLE II. Effect of DEAE-Dextran on Plaque Diameter and Efficiency of Plating as a Function of Plaque Development Period.

Virus	Plaque development period (days)							
	Mean plaque dia (mm)*				Mean relative e.o.p.†			
	2	3	4	5	2	3	4	5
Brunhilde wild	3.0	5.0	7.0	10.0	1.0	1.2	1.4	1.6
Mahoney "	2.5	5.0	8.0	12.0	1.0	1.0	1.2	1.0
Brunhilde mutant m_α	1.5	5.0	9.0	12.0	.1	.8	1.0	1.0
" " m_π	1.0	4.5	10.0	14.0	.2	.9	1.0	1.0
" " m_ϵ	2.0	7.0	12.0	14.0	.2	1.0	1.0	1.0
Slow Mahoney mutant	1.5	4.0	10.0	13.0	.6	.9	1.0	1.0
ECHO 5, wild	1.5	2.5	8.0	10.0	.1	.8	1.2	1.0
" 8, "	2.0	3.0	8.0	12.0	.1	.6	1.0	1.0
" 11, "	1.5	4.0	9.0	12.0	.2	.8	1.0	1.4
" 26, "	1.0	4.0	10.0	12.0	.1	.5	1.4	1.2

* Each plaque diameter is an average of 25 well-separated plaques from 10 plates.
† E.o.p.'s are plaque titers on plates receiving agar-hcAE$_m$ divided by those on plates receiving agar-hcAE$_m$ plus 100 μg/ml DEAE-dextran.

ment period. Experiments were made to test the effect of DEAE-dextran on plaque size and e.o.p. of polio- and echoviruses in relation to their plaque development period. Plaque diameters shown in Table II are those on plates receiving agar-hcAE$_m$ plus 100 μg/ml DEAE-dextran and stained at various intervals, whereas relative e.o.p.'s have been calculated by dividing the plaque titers on control plates receiving agar-hcAE$_m$ by those on plates receiving 100 μg/ml DEAE-dextran plus agar-hcAE$_m$ overlay. Results indicate that e.o.p. was dependent on the number of days in which plaques were revealed.

Effect of DS on viral CPE. Since plaque diameters of poliovirus mutants m_{alpha}, m_{pi}, $m_{epsilon}$, and slow Mahoney and ECHOs 5, 8, 11, and 26 were greatly increased when inhibition by naturally-occurring sulfated polysaccharide in agar was counteracted by DEAE-dextran, the rate of multiplication of these viruses in monkey kidney cells under liquid medium in the presence of 100 μg/ml DS (a synthetic sulfated polysaccharide) and in agar extract was tested and compared with that of wild-type polioviruses. Inoculum contained 1,000 plaque-forming units (PFU)/ml of each virus. Results in Table III indicate that CPE of the polio mutants and echoviruses was much slower in both DS and agar extract as opposed to wild-type polioviruses which were unaffected by either substance. In additional experiments in which 10^6 PFU/ml were contained in the inoculum no

TABLE III. Effects of Agar Extract and Dextran Sulfate on Cytopathic Effect of Polio- and Echoviruses in Cultures of Primary Monkey Kidney Cells.

Virus	Mean days required for completion of CPE*		
	A	B	C
	hcAE$_m$	hcAE$_m$ + agar extract†	hcAE$_m$ + dextran sulfate
Brunhilde wild	3	3	3
Mahoney wild	3	3	3
Brunhilde mutant m_α	4	6	8
" " m_π	4	6	8
" " m_ϵ	4	7	7
Slow Mahoney mutant	4	6	7
ECHO 5, wild	5	7	8
" 8, "	4	7	7
" 11, "	5	8	8
" 26, "	5	7	7

* Mean days required for complete CPE were calculated from results from 10 tubes per medium per virus.
† 50%, by volume, of medium was agar extract.

TABLE IV. Effect of Various Concentrations of Dextran Sulfate on Plaque Diameters of Polio Mutants and Echoviruses.

Virus	Mean plaque diameter (mm)* Concentration of dextran sulfate in overlay (μg/ml) 0	50	100	200	400
Brunhilde mutant m_α	1.0	1.0	.5	.1	NV†
" " m_π	1.0	1.0	.3	.1	NV
" " m_ϵ	1.0	1.0	.4	.1	NV
Slow Mahoney mutant	2.0	1.5	.6	.2	NV
ECHO 5, wild	1.0	1.0	.4	.1	NV
" 8, "	1.0	1.0	.4	.1	NV
" 11, "	1.0	1.0	.5	.1	NV
" 26, "	1.0	1.5	.6	.1	NV

* Each mean plaque diameter is based on an average of 30 plaques from 3 plates.
† Not visible.

delay in the appearance of CPE was observed in the presence of the same concentration of DS.

Experiments were also made with *m* mutants to determine whether or not these inhibitors would select a population of viruses resistant to agar extract or DS. Each of the mutants grown in agar extract and in 100 μg/ml DS produced minute plaques under $hcAE_m$-DS overlay.

Effect of DS on plaque diameter. The effect of DS on plaque formation by poliovirus *m* mutants and ECHOs 5, 8, 11 and 26 was tested. Results of these tests appear in Table IV. Plaque sizes decreased with increasing concentrations of DS (the opposite effect was observed with DEAE-dextran); at 400 μg/ml no plaques were discerned.

In other experiments in which higher concentrations of *m* mutants were plated (it was possible to score approximately 120 plaques per plate) a slightly larger plaque (2.5 mm diameter) was occasionally observed; however when replated in the presence of 100 μg/ml DS these viruses again produced plaques about 2.5 mm in diameter, indicating a moderate resistance to DS. No large plaques were formed by any of the *m* mutants in the presence of DS.

Effects of DS on rate of appearance of progenies. In other experiments we noted that DS did not affect plaque size or rate of multiplication of wild-type polioviruses. Experiments were made to determine the effect of DS incorporated into the virus growth medium on rate of multiplication of poliovirus *m* mutants. Monkey kidney plates were inoculated with approximately 1,000 PFU/ml; half of the plates received $hcAE_m$ medium without agar and half received the same medium plus 100 μg/ml DS. Samples taken at various intervals were harvested by freezing and thawing then were titrated by plaque method. Results are given in Fig. 2. Yields of the mutants were 1-2 logs lower when DS was added to the medium.

Comparison of effects of L-cystine and DEAE-dextran on plaque diameters. In a study of cystine requirements for normal poliovirus action on monkey kidney cells Dubes(13) reported that the cytopathogenic action of strains Akron, Brooks and Mabie was considerably delayed when cystine was omitted from the medium and further noted that larger plaques were formed by these strains in the presence of 0.5 mM cystine.

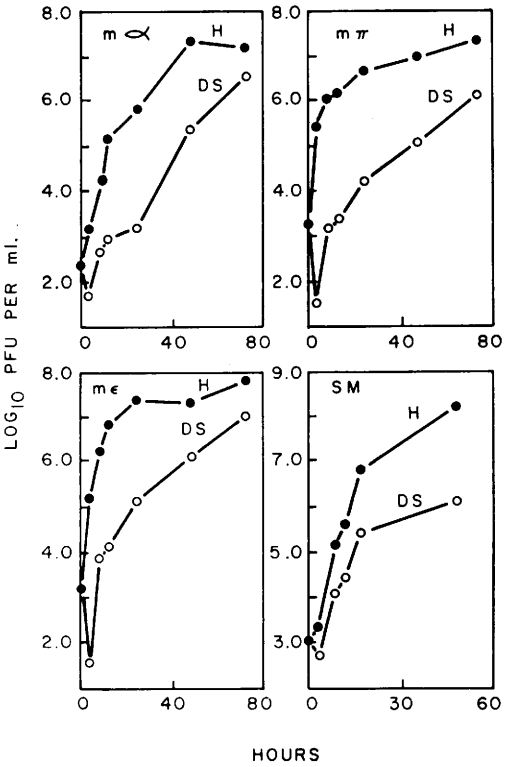

FIG. 2. Effect of dextran sulfate on rate of multiplication of poliovirus minute-plaque mutants m_{alpha}, m_{pi}, $m_{epsilon}$, and slow Mahoney. H refers to medium $hcAE_m$; DS is $hcAE_m$ + 100 μg/ml dextran sulfate.

TABLE V. Effects of L-Cystine and DEAE-Dextran on Plaque Diameters of Polio- and Echoviruses.

Virus	Mean plaque diameter (mm)*			
	Agar-AE_m (0 cystine)	Agar-AE_m + .2 mM cystine†	Agar-AE_m + 1.0 mM cystine	Agar-$hcAE_m$ + 50 μg/ml DEAE-dextran
		No change in size		
Mahoney wild	4.0	5.0	4.5	4.0
Charbonneau wild	3.5	3.2	3.0	3.5
Theiler's Lansing wild	2.5	2.0	2.0	2.0
		Moderate change in size		
Brunhilde wild	3.0	5.0	5.0	5.0
Akron "	2.5	4.0	4.0	4.0
		Marked change in size		
Brunhilde mutant m_α	1.0	1.3	1.2	6.0
" " m_π	.6	.6	.7	5.0
" " m_ϵ	.8	.8	.8	7.0
Slow Mahoney mutant	1.2	1.4	1.5	8.0
ECHO 5, wild	1.0	2.2	2.5	6.0
" 8, "	2.0	3.0	3.0	5.2
" 11, "	1.0	2.4	2.8	4.5
" 26, "	1.0	3.2	3.0	5.8

* Plaque diameter for each virus is the mean from 30 well-separated plaques on 6 plates.
† Agar-AE_m + 0.2 mM cystine is the same as agar-$hcAE_m$.

Experiments were made to compare the effects of L-cystine and DEAE-dextran on 4 *m* mutants of poliovirus, certain wild-type polioviruses, and echoviruses 5, 8, 11, and 26. Results of these experiments shown in Table V provide evidence that concentrations of L-cystine ranging from 0 to 1 mM did not appreciably affect the plaque size of the poliovirus mutants, although plaque diameters of Brunhilde and Akron wild-types and ECHOs 5, 8, 11, and 26 were increased about equally with 0.2 mM and 1.0 mM L-cystine. DEAE-dextran caused a great increase in plaque diameters of the poliovirus *m* mutants and echoviruses tested. Wild-types of Mahoney, Theiler's Lansing and Charbonneau were not affected by either DEAE-dextran or L-cystine.

Discussion. Inhibition of sulfated polysaccharides as reported herein may be, as was concluded by Young and Mora(14) and Liebhaber and Takemoto(15), due to interaction between oppositely charged sites on the inhibitor and the virus. Formation of the virus-inhibitor complex has been shown to be reversible with dengue(16) and EMC(4) viruses by dilution of the mixture.

In speculation as to why the poliovirus mutants studied herein were inhibited by natural and synthetic polysaccharides while their wild-types were not similarly affected, we note that inhibition was acquired by the *m* mutants of Brunhilde as a result of passaging the wild-type virus (grown on primary monkey kidney cells) in established lines of monkey kidney cells which required 4% calf serum in the maintenance overlay; no inhibitor (agar) was present in the liquid growth medium. The slow Mahoney mutant also acquired inhibition by passaging the wild-type virus in HeLa cells maintained in a serum-containing medium. It seems highly possible that the contribution of host-cell materials and/or serum proteins from the maintenance medium account for a change in the mutant resulting in its inhibition by sulfated polysaccharides.

Evidently the prototype echoviruses studied were somewhat inhibited by sulfated polysaccharides. The mechanism resulting in increased plaque diameter of these viruses by the addition of DEAE-dextran to the overlay does not appear to be comparable to that by which L-cystine increased plaque diameter, as we have shown in unpublished experiments that L-cystine acts intracellularly to increase viral synthesis, and we have further shown that L-cystine does not inhibit adsorption of echoviruses to the cells. Liebhaber and Takemoto(15) reported inhibition of ad-

sorption by DS in the case of EMC virus. The action of DEAE-dextran in removal of inhibition by binding of the sulfated polysaccharides is probably entirely extracellular and unrelated to the action of L-cystine.

Summary. DEAE-dextran was shown to increase plaque diameter of poliovirus *m* mutants m_{alpha}, m_{pi}, and $m_{epsilon}$, and slow Mahoney and wild-type echoviruses 5, 8, 11, and 26, but it did not affect plaque diameter of wild-type polioviruses tested. DS and agar extract slowed completion of CPE by poliovirus *m* mutants and echoviruses; however, wild-type polioviruses were not affected. The increase or decrease in plaque diameters of poliovirus mutants and echoviruses was dependent on increased concentrations of DEAE-dextran and DS, respectively. The effect of DEAE-dextran on e.o.p. of poliovirus mutants and echoviruses was related to their plaque development period. L-cystine did not affect plaque size of poliovirus *m* mutants nor wild-types of Mahoney, Theiler's Lansing, and Charbonneau; however, it increased plaque diameters of Brunhilde and Akron wild-types and echoviruses 5, 8, 11, and 26.

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Intercellular Forces and Separation Energy of Red Cells Sludged by Contrast Medium.* (30060)

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Knisely and his coworkers showed that sludging of red blood cells occurs in over 100 pathological conditions, or, alternatively, in the presence of excess of a contrast medium such as sodium diatrizoate(1). Bernstein and his coworkers enumerated possible intercellular forces of repulsion and attraction which could be altered with the occurrence of such red cell sludging(2), while Bloch and his coworkers reported that this sludging involves the formation of a sticky coating around the sludged red cells(3). This coating is observable with the electron microscope but usually not with the light microscope. The data given here were obtained by applying Berwick and Coman's method(4) to measurement of the adhesion forces and energy of sludged red cells, and these data are combined with microelectrophoresis findings to show that viscosity

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