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Mechanism of Enhancement of Virus Plaques by Cationic Polymers

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It has been assumed that plaque enhancement by cationic polymers is due to their binding of sulfated polysaccharides in agar. However, viruses that are enhanced by cationic polymers, diethylaminoethyl-dextran, and protamine were found *not* to be inhibited by polyanions in agar under the usual overlay conditions. In the case of adenovirus, enhancement by protamine seems to be due to the protamine serving as a source of arginine; enzymes released from the cultured cells digest the protamine and provide a reservoir of arginine for the cells. Other viruses (herpes and echovirus types 3, 4, 5, and 6) known to be susceptible to agar inhibitors were found to be enhanced by cationic polymers even under starch gel and methylcellulose overlays, which are free of polyanions. Since cationic polymers enhance the diffusion of virus through agar or starch gel, plaque enhancement seems to be the result of the gel becoming positively charged so that viruses can move effectively through them. The observation that starch gel and methylcellulose enhance plaque formation with viruses known to be inhibited under agar was also reinvestigated. When the consistency of the agar gel was reduced to the same viscosity of starch gel and methylcellulose overlays, the same plaque counts and sizes were observed under all three overlays.

The virus literature contains many reports, comprehensively reviewed by Takemoto (20), attributing the inhibition of animal viruses under agar overlays to the anionic, sulfated polysaccharides in agar. This inhibitory action is believed to result from the binding of virus to the polyanions, thus preventing virus adsorption to cells (1, 4, 13, 14, 17, 26, 28). Anionic polymers have also been reported to enter cells and interfere with virus replication (19, 23). Certain viruses can be enhanced by incorporation of cationic polymers in overlays, supposedly by neutralizing anion polymers in the overlay (12). Solidifying agents such as methylcellulose or starch gel, which are free of anionic polymers, have been shown to enhance plaque formation of viruses suppressed under agar (8, 15, 18, 24).

There seem to be a number of peculiarities in the above reports. First, although agar extracts prevent virus adsorption, they have to be used in concentrations much higher than that naturally present in the overlay (4, 22, 23). Second, certain viruses (vaccinia and measles) which are enhanced under starch gel (8) or methylcellulose (15) overlays free of sulfated polysaccharides are not enhanced under agar overlays containing cationic polymers. Certain viruses (LSc poliovirus, S-mengo virus, influenza virus type B, and others) that are enhanced under agar by protamine sulfate are actually inhibited under starch gel and methylcellulose overlays (3, 6). Third, although the synthetic polyglucose compound, dextran sulfate, interferes with the replication of virus in cells (23), this compound has not proven to be a suitable model for the natural polyglucose compounds in agar (3). Consequently, an investigation was made of the mechanism of enhancement of virus plaques by cationic polymers.

MATERIALS AND METHODS

Monkey kidney (MK) cells. Kidneys from immature green (vervet) monkeys were trypsinized and grown in 2% calf serum in Melnick's medium A.

Viruses. This study included viruses that have been reported to be sensitive to sulfated polyanions contained in agar. These were adenovirus (SV15), the large plaque-forming strain of JES herpesvirus (10, 15), and echovirus types 3, 4, 5, and 6. All viruses were grown in a single cycle in MK cells maintained with Melnick's medium B without serum and were stored at −90 C.

Virus assays. The plaque-forming unit (PFU) method was used. Drained MK cultures in 30-ml bottles with a 12 cm² surface were inoculated with viruses at an input of about 25 to 50 PFU/culture

contained in 0.1-ml volume. After 1-hr adsorption at 37 C, cultures were overlaid with 5 ml of the following medium: Earle's salt solution, 0.4% $NaHCO_3$, 1:60,000 neutral red, Eagle's basal medium amino acids, and 1.5% agar (Difco). Any variations from this overlay are indicated in the Results.

Solidifying agents. Bacto-agar (Difco) is rich in sulfated polysaccharides. This agar will form a precipitate with either protamine sulfate or diethylaminoethyl (DEAE)-dextran at an agar concentration as low as 0.015%. Methylcellulose (4,000 cps) and commercial hydrolyzed starch gel (Mann) were prepared as described (8, 15). Regardless of the solidifying agent used, all overlay media contained the components described in the preceding paragraph.

Additives to overlay medium. Protamine sulfate (Salmine) was obtained from the Nutritional Biochemicals Corp.; DEAE-dextran, from Pharmacia, Upsala, Sweden.

Proteolysis of protamine sulfate. A 9-ml stock of 1% protamine sulfate in distilled water was adjusted to *p*H 8 with 1 ml of tris(hydroxymethyl)aminomethane (Tris)-buffered saline-NaOH containing 1% trypsin. The sample was incubated at 37 C for 2 to 3 hr, at which time the protamine suspension cleared. At this point it no longer precipitated dextran sulfate, and it was assumed that the protamine had been digested into its amino acid components. Control samples of protamine with Tris buffer but without trypsin, and a trypsin control without protamine, were also incubated as described. All samples were then boiled for 15 min to inactivate the trypsin, and were used as additives for overlays by diluting them 1:25 in agar.

RESULTS

Effects of agar extracts on cell susceptibility. The viruses used, herpes, adeno, and echovirus types 3, 5, and 6, have been shown to be enhanced under agar overlays by cationic polymers (9, 12, 24, 25, 29), and thus it had been assumed that they are inhibited by sulfated polysaccharides in agar. Recently, we found that LSc poliovirus, which had also been reported to be sensitive to high concentrations of anionic polymers (20), was not sensitive to agar extracts (Wallis and Melnick, *in press*). This led to an investigation of the effects of agar extracts on the above viruses.

Agar overlays were made in bottles without cells. When the agar had solidified, fluids from normal MK cultures maintained for 3 days with fluid medium were added to the agar surfaces, and the bottles were incubated at 37 C for 4 days. The fluids were then recovered from the agar surfaces and were used as diluents for the viruses. As a control, fluids that had never been exposed to agar were used. It was found that herpesvirus, adenovirus, and the echoviruses were adsorbed to cell monolayers to the same degree whether diluted in agar-treated fluids or in control fluids. Thus, agar under conditions simulating a natural overlay does not release virus inhibitors in concentration sufficient to interfere with virus adsorption. Studies reported by others in which such extracts inhibited virus adsorption were carried out with purified extracts, or concentrated extracts, used in relatively high concentration (1, 14, 17, 22, 23).

It has been suggested that polyglucose compounds in agar may enter cells and abort virus replication (19, 23). However, the following experiment showed that agar products under natural overlay conditions do not interfere with virus growth. Normal MK cultures were maintained for 4 days with (i) fluid medium as used in agar overlays, and (ii) complete agar overlays with 0.75% agar ("sloppy agar"). Cultures were maintained for 4 days at 37 C, and then the fluid medium and the sloppy agar were decanted. The cultures were rinsed twice with Tris buffer and were then used for inoculation of the viruses listed above. After 1-hr adsorption at 37 C, all cultures were overlaid with complete agar medium. Cultures maintained for 4 days with fluid medium yielded essentially the same number of plaques as did the cultures maintained with agar for 4 days. Thus, agar products did not diffuse into the cells and prevent plaque formation of herpesvirus, adenovirus, or echovirus. The workers who previously demonstrated virus inhibition with polyglucose compounds used a synthetic product. Since then, it has been well documented that natural agar polyglucose compounds do not inhibit viruses to the same extent as do synthetic polyanions (3; Wallis and Melnick, *in press*).

Since agar components do not directly inhibit plaque formation, reasons were sought for inhibition of viruses under agar and for their enhancement by cationic polymers. The above viruses were used as model agents, since several workers have concluded that they are sensitive to agar inhibitors (8, 9, 12, 14, 19, 21–27).

Plaque enhancement of adenovirus by protamine. The findings of Liebhaber and Takemoto (12) led Tytell et al. (25) to use protamine to overcome the inhibitory effects of agar on adenovirus. Since adenovirus does not produce plaques under methylcellulose or starch gel overlays, even when cationic polymers are added to overlays, the effects of cationic polymers on adenovirus in a system free of sulfated polymers could not be tested. However, if the integrity of the cells under agar could be maintained by binding anionic polymers with protamine (25), enhanced plaque formation of this virus should also be obtained by the use of DEAE-dextran. As protamine is composed predominantly of arginine, consideration was also given to the findings of Rouse et al.

(16), who showed that adenovirus replication is enhanced by this amino acid. Since MK cells synthesize a large amount of proteolytic enzymes (2), it is possible that during the incubation period the cellular enzymes digest protamine into its amino acid components, providing the cells with a rich source of arginine. The following experiment was performed to investigate this possibility.

Adenovirus was inoculated onto drained MK cultures at an input of 25–50 PFU/cultures. After 1-hr adsorption at 37 C, the cultures were overlaid with the standard medium plus the following additives: (i) none; (ii) DEAE-dextran (100 to 800 μg/ml); (iii) protamine (400 μg/ml); (iv) digested protamine (400 μg/ml); (v) 1% trypsin; and (vi) arginine (300 μg/ml; the concentration present in 400 μg of protamine per ml).

Overlays containing DEAE-dextran failed to enhance the plaque formation of adenovirus (Table 1). On the other hand, protamine overlays produced increased plaque counts and sizes. Digested protamine and arginine overlays also enhanced the counts and sizes of adenovirus plaques to the same degree as protamine overlays. Thus, it appears that the enhancement of adenovirus by protamine is due to the arginine component of protamine.

To determine whether intact protamine could function as the enhancing agent, a sample of the trypsin-digested protamine was serially diluted and tested for its capacity to precipitate dextran sulfate from solutions containing 1, 0.1, and 0.01% of the anionic polymer. The digested protamine failed to precipitate dextran sulfate at any concentration. On the other hand, nondigested protamine precipitated 1% dextran sulfate at a dilution of 1:40, and 0.01% dextran sulfate at a dilution of 1:640.

The question was then asked whether cellular enzymes digest protamine under the conditions used in the overlay. Normal MK monolayers were maintained for 48 hr with the same medium used for overlays except that the agar and neutral red were omitted. The fluid medium was removed and protamine was added to the cell-free fluids to give a final concentration of 400 μg/ml. As a control, protamine was added to bottles containing fresh medium but no cells. The samples were incubated overnight at 37 C, and the next day the samples were serially diluted and tested against dextran sulfate. The suspension of protamine in the fluids obtained from normal MK cells had cleared and did not react with dextran sulfate even when tested undiluted. On the other hand, protamine in freshly prepared medium held without cells at 37 C was still turbid, and upon serial dilution precipitated dextran sulfate even after the sample was diluted to 1:160. The rapid

TABLE 1. *Effects of cationic polymers on plaque formation by adenovirus*

Additive in overlay	Concn	No. of plaques/0.1 ml on 5th day (avg of four cultures)	Avg size of plaques
	μg/ml		*mm*
None		12	0.3
DEAE-dextran	800	14	0.3
	400	17	0.3
	200	10	0.3
	100	19	0.3
Protamine sulfate	400	75	1.5
Digested protamine[a]	400	82	1.5
Arginine	300	87	1.5

[a] The trypsin solution used to digest the protamine had no enhancing effect when added to the overlay.

digestion of protamine in agar overlay could be followed by observing the turbidity of overlays containing protamine sulfate. The turbidity was steadily reduced so that the overlay was clear after 4 to 5 days.

When protamine that had been digested by cell enzymes was used as an additive in adenovirus overlays, plaque enhancement was the same as that in corresponding cultures overlaid with untreated protamine.

Plaque enhancement of herpesvirus. Herpesvirus has been reported (8, 15, 21, 23, 24) to be sensitive to sulfated polysaccharides in agar. The basis of this statement is found in the relief of the inhibitory effects of agar by the addition of protamine or in the use of starch gel or methylcellulose overlays. However, no experiments are known to us in which the effect of protamine on the plaque formation of herpesvirus under methylcellulose or starch gel overlays has been studied.

In preliminary experiments, we confirmed the finding that herpesvirus is enhanced by protamine but not by DEAE-dextran (21). We also noted that herpesvirus is enhanced by intact protamine and not by digested protamine. To evaluate the effects of cationic polymers on herpesvirus, overlays were made by use of different solidifying agents with various concentrations of protamine (Table 2).

Although protamine enhanced the plaque formation of herpesvirus under agar, it also enhanced virus replication under starch gel and methylcellulose overlays, which are free of sulfated anions. Although plaques were somewhat larger in starch gel and methylcellulose overlays in the absence of protamine when compared to their size under agar overlays, sulfated polysaccharides in agar cannot be the cause of the

TABLE 2. *Effects of different solidifying agents and protamine sulfate on plaque formation of herpesvirus*

Solidifying agent	Protamine sulfate concn	No. of plaques/ 0.1 ml on 5th day (avg of four cultures)	Avg size of plaques
	μg/ml		*mm*
Agar (1.5%)	0	20	1.5
	400	51	4.5
	100	37	2.5
	25	18	1.5
Methylcellulose (1%)	0	42	2.5
	100	2	0.5
	25	67	6.5
	5	49	4.5
Starch gel (10%)	0	31	2.5
	100	7	0.5
	25	54	7.0
	5	41	3.5

poor formation of plaques. Starch gel and methylcellulose are less viscous than 1.5% agar, which might account for more rapid diffusion of virus and large plaques. Experiments carried out with agar at a concentration of 0.4%, which yields the same gel viscosity as that of starch gel or methylcellulose, yielded herpes plaques of the same size as those in cultures overlaid with 10% starch gel or 2% methylcellulose. The data do not eliminate the possibility that lowering the viscosity by dilution may not dilute out some undefined inhibitor for herpesvirus present in the agar. Such an inhibitor cannot be sulfated polysaccharide, for repeated tests comparing agar rich in sulfated polysaccharides with agarose (which is free of such material) gave the same plaque number and size with herpesvirus.

Plaque enhancement of echoviruses. The replication of a number of echoviruses (types 2, 3, 5, 6, 9, 13, 20, and 26) under agar has been shown to be enhanced by DEAE-dextran (9, 12) or by protamine (29). Thus, the mechanism of plaque enhancement with these viruses differs from that with adenovirus and herpesvirus, which are not enhanced by DEAE-dextran. Also, in contrast to adenovirus, the echoviruses could not be enhanced by digested protamine. The following experiments were performed to determine the effects of cationic polymers on echoviruses under solidfying agents free of sulfated polymers.

Echovirus types 3, 5, and 6 were inoculated into drained MK cultures as described above. After 1-hr adsorption at 37 C, the following overlays were added: 1.5% agar, 10% starch gel, and 1% methylcellulose, each overlay containing the salts and nutrients described under Materials and Methods. In addition, representative overlays contained different concentrations of cationic polymers (Table 3). The findings are shown only for echovirus type 6 since echovirus types 3 and 5 gave essentially the same results.

Echovirus was enhanced under agar overlays with either protamine sulfate or DEAE-dextran. However, these cationic polymers also enhanced virus plaques under methylcellulose or starch gel overlays. Thus, under agar echoviruses do not seem to be inhibited by sulfated polysaccharides, since plaque formation was similar under agar, methylcellulose, and starch gel overlays, and enhancement with cationic polymers was the same for each of the three overlays. Enhancement of the virus could be observed only with lower concentrations of cationic polymers under starch gel or methylcellulose, for higher concentrations of these polymers are toxic in that the cells may be altered and virus plaque formation inhibited. In the case of agar, large concentrations of cationic polymers are needed for plaque enhancement, since a major portion is bound by the sulfated polyanions in agar. Thus, in agar, cationic polymers must be present in a concentration of 400 μg/ml to neutralize the sulfated anions present and still permit free cationic polymers

TABLE 3. *Effects of cationic polymers on echovirus type 6*

Concn of cationic polymers	No. of plaques/0.1 ml on 5th day (avg of four cultures)[a]					
	Under agar overlay plus		Under starch gel overlay plus		Under methylcellulose overlay plus	
	Protamine	DEAE-dextran	Protamine	DEAE-dextran	Protamine	DEAE-dextran
μg/ml						
400	92 (2.5)	99 (3.5)	Toxic	Toxic	Toxic	Toxic
100	41 (1.5)	49 (1.5)	6 (0.3)	1 (0.3)	0	0
25	27 (1.0)	21 (1.0)	116 (3.5)	101 (3.5)	99 (3.5)	118 (3.5)
5	ND	ND	79 (2.5)	70 (2.5)	64 (2.5)	60 (2.5)
1	ND	ND	41 (1.0)	30 (1.0)	22 (0.5)	30 (0.5)
0.2	ND	ND	21 (0.5)	30 (0.5)	20 (0.5)	27 (0.5)

[a] Numbers in parentheses represent average plaque size (millimeters). In a control experiment (overlays without cationic polymers), agar yielded 25 plaques (per 0.1 ml, 5th day); starch gel, 31; methylcellulose, 17; plaque sizes were 1.0, 0.5, and 0.5 mm, respectively.

to diffuse into the cells. Other workers have reported enhancement of virus replication under agarose or other purified agars with lower concentrations of cationic polymers; this can be accounted for by the fact that these agars contain fewer sulfated anions.

Effects of protein nutrients in agar on the activity of cationic polymers. Other workers (9, 22) reported that the same viruses enhanced in one study with protamine (29) were enhanced in their study with DEAE-dextran (50 μg/ml). The difference in the results probably lies in the different protein-rich additives used, for they may lower the activity of the cationic polymers. Thus, 1% fetal calf serum (22), 0.1% bovine albumin-yeast extract (2, 22), and 0.1% skim milk (29) have been used in the above studies. To determine the effects of these nutrients on the activity of protamine and DEAE-dextran, the following experiments were performed.

Echovirus type 6 was inoculated into drained MK monolayers at an input of 25 to 50 PFU/culture. After 1-hr adsorption at 37 C, the cultures were overlaid with the agar medium described under Materials and Methods, with one of the following additives: (i) none; (ii) 0.1% bovine albumin (Armour)-0.1% yeast extract (Difco); (iii) 1% bovine fetal serum; (iv) 1% fetal serum-0.1% bovine albumin-0.1% yeast extract; and (v) 0.1% skim milk. Duplicate cultures containing these additives were also treated with protamine sulfate and DEAE-dextran at the concentrations used in the above studies.

It is evident (Table 4) that proteinaceous nutrients can affect the activity of cationic polymers. Echovirus type 6 was enhanced markedly by protamine or DEAE-dextran in protein-free overlays or in overlays containing skim milk and protamine, whereas DEAE-dextran did not enhance the plaque formation of this virus in the presence of skim milk. Thus, it seems that skim milk binds DEAE-dextran and prevents its activity.

Effects of cationic polymers on the diffusion of echovirus type 4 through overlay gels. Recently, Conant and Barron (5) reported that echovirus type 4 (DuToit) failed to produce precipitin lines in agar gel diffusion tests and attributed this inhibition to the sulfated polymers present in the agar. They subsequently showed that protamine sulfate enhanced virus plaques under agar; similarly, when protamine sulfate was included in gel diffusion tests, enhanced precipitin lines were manifest. They concluded that agar-sulfated inhibitors were responsible for both effects. In our hands, cationic polymers enhanced DuToit virus not only under agar but also under starch gel and methylcellulose overlays. Therefore, it appears that cationically charged gels will allow virus to diffuse through them more rapidly than through gels devoid of these positively charged polymers.

The following experiment was performed to determine the effects of cationic polymers on the diffusion of echovirus type 4 through solidifying agents. Normal, uninoculated MK cultures were overlaid with complete agar and starch gel medium with and without cationic polymers. When the gels had solidified, a virus stock containing 10^6 PFU/ml was streaked across the surface of the gels with a loop, with each culture receiving about 5×10^4 PFU of virus. Cultures were then placed at 37 C to allow the diffusion of the virus through the solidifying agents to the cell monolayer. Plaques were counted on the 10th day (Table 5). Diffusion of DuToit virus through agar and starch gel in the absence of cationic polymers

TABLE 4. *Effects of proteinaceous nutrients in agar medium on activity of cationic polymers on echovirus type 6*

Nutrients	No. of plaques/0.1 ml on 5th day (avg of four cultures)[a]		
	No additive	Protamine sulfate (400 μg/ml)	DEAE-dextran (100 μg/ml)
None	37 (1.0)	69 (2.0)	61 (2.0)
Skim milk (0.1%)	10 (0.5)	99 (2.0)	14 (0.5)
Yeast extract (0.1%) + bovine albumin (0.1%)	25 (1.5)	78 (2.0)	64 (1.5)
Fetal calf serum (1%)	42 (0.5)	75 (2.5)	60 (1.5)

[a] Numbers in parentheses represent average plaque size (millimeters).

TABLE 5. *Effects of cationic polymers on the diffusion of echovirus type 4 through overlay gels*

Solidifying agent	Total no. of PFU that diffused through agar and reached cell monolayer to form plaques by the 10th day		
	No additive	Protamine sulfate[a]	DEAE-dextran[a]
Agar (0.75%)	2	87	89
Starch gel (10%)	0	69	101

[a] In agar, 200 μg/ml; in starch gel, 50 μg/ml.

was *inefficient*, since only 2 PFU was evident after 10 days of incubation. On the other hand, virus diffused more rapidly through either gel in the presence of cationic polymers.

Cultures were observed for an additional 5 days. Overlays free of polycations manifested only an occasional plaque by the 15th day, whereas overlays containing cationic polymers yielded 50 to 100 additional plaques on the 12th day and, by the 15th day, the cultures were confluent. In repeat experiments, with 10 times the inoculum on top of the agar or starch surface, control cultures (both agar and starch gel) had an average of 12 plaques by the 10th day, whereas cultures containing cationic polymers were already confluent because of the multitude of tiny plaques that appeared.

Control cultures inoculated with echovirus type 4 directly on the monolayer and overlaid with the different solidifying agents with and without cationic polymers all yielded approximately the same number of plaques on the 10th day; however, with cationic polymers, the plaques were larger and more discrete. In the case of echoviruses, plaque counts under overlays free of cationic polymers approached those of cultures overlaid with polycations, if cultures were maintained for 10 days. Thus, although plaque counts of echovirus type 4 were 50% less in control cultures than in overlays with polycations on the 5th day, by the 10th day similar counts were evident. Since cationic polymers enhance the plaque size of viruses, plaques can be observed and counted earlier.

Discussion

Previous investigators had assumed that viral enhancement by cationic polymers was due to the neutralization of anionic polymers in the agar overlay; however, we have found that cationic polymers may also enhance viruses under overlays free of sulfated polyanions. Thus, factors other than sulfated polysaccharides in agar seem to be responsible for inhibiting viral replication.

A search of the literature has revealed a number of discrepancies. On the basis of studies with dextran sulfate, a synthetic polyglucose compound (19, 21, 22), a number of workers have concluded that sulfated polyglucose compounds naturally in agar are responsible for viral inhibition. However, other workers have shown that dextran sulfate has not always proved a satisfactory model for agar polyanions. Thus, Takemoto and Fabisch (22) showed that synthetic dextran sulfate added to agar inhibited herpesvirus, whereas natural polysaccharides from agar did not. Campbell and Colter (3) showed that M-mengovirus which was enhanced by protamine was also enhanced by dextran sulfate. Our own studies had shown that poliovirus (LSc strain) was sensitive to dextran sulfate and not to agar extracts (Wallis and Melnick, *in press*).

Tytell and co-workers (24, 25) reported that protamine maintained the integrity of cell monolayers under agar over prolonged periods of time, and enhanced the plaque formation of herpesvirus, adenovirus, and parainfluenza virus. The data obtained in the present study indicate that the plaque enhancement of adenovirus is not due to protamine binding of the anionic polymers in agar, but to protamine serving as a source of arginine. When cellular enzymes digest protamine, arginine is released and functions as an enhancing factor. In the case of herpesvirus, which is enhanced by protamine sulfate and not by DEAE-dextran, it is likely that the protamine neutralizes the inhibitory effects of some cell products (probably proteolytic enzymes), since herpesvirus is also enhanced by protamine under overlays free of sulfated polyanions. The enhancement of plaque formation by cationic polymers—previously believed to be accomplished through their binding of sulfated polyanions—apparently comes about through the effect of these polymers upon the diffusion of virus to cells. Recently, rabies virus (11), rubella virus (27), and poliovirus (Wallis and Melnick, *in press*) have been found to be enhanced in fluid cultures by cationic polymers.

The variations reported by a number of laboratories on the efficiency of protamine or DEAE-dextran in enhancing virus plaques (8, 11, 28) were found to be due to the protein-containing nutrients used in agar overlays. Thus, the activity of DEAE-dextran is reduced considerably by skim milk.

The increase in plaque sizes of viruses under purified agar has been attributed to the reduction of sulfated polysaccharides present in the overlays (3). However, purified agars and agarose are more alkaline than crude agars, and their overlays maintain higher *p*H levels than does conventional agar. Thus, increased plaque formation of viruses under these purified agars may be due to elevated *p*H rather than to the absence of sulfated polyanions. Campbell and Colter (3) have shown that S-mengovirus requires protamine to produce plaques under conventional agar; under purified agar, this virus produces small plaques without protamine. Under methylcellulose, the virus is also inhibited (3). We have shown that methylcellulose overlays are more acid than agar overlays (Wallis and Melnick, *in press*). Thus, it is likely that the difference obtained between conventional agars and purified agars may be a matter of hydrogen ion concentration. In this connection S-mengo,

M-mengo, and L-mengovirus strains produce plaques of the same size under conventional agar containing sulfated polymers as under methylcellulose, which is free of this polyglucose (3). The problem of inhibition seems related to cell environment existing under solidifying agents rather than to the presence of sulfated polysaccharides.

Others have speculated as to the possibility that sulfated polymers in agar do not necessarily play a major role in inhibition of viruses. Campbell and Colter (3) asserted that, in addition to anionic polymers, a second factor in agar is present which is capable of inhibiting viruses, and that both factors are relieved by protamine. Craighead (7) recently showed enhancement of virus infection in mice by the use of DEAE-dextran. Although his work was based on the assumption that natural sulfated polyanions in animal tissues may inhibit virus growth, the effects of DEAE-dextran on enhancing infectivity in vivo seemed due to some other mechanism (7).

From the results shown in this study, it is apparent that viruses diffuse through solidifying agents (agar, methylcellulose, or starch gel) more efficiently in the presence of cationic polymers than in their absence. Thus, viruses seem to move more rapidly along or through a gel that is positively charged.

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