Diethylaminoethyl-Dextran Enhancement of Interferon Induction by a Complexed Polyribonucleotide (34684)

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(Introduced by Maxwell Finland)

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Cells exposed to the ribonucleotide complex of the homopolymers of inosinic acid and cytidilic acid (I:C) will resist direct challenge with virus and also induce the production of assayable interferon (1). Furthermore, these biological effects of I:C can be enhanced by the presence of polycationic substances (2, 3) (also personal communications from E. Falcoff and R. Perez-Bircoff (17); and from A. Billiau, C. E. Buckler, F. Dianzani, C. Uhlendorf, H. B. Levy, and S. Baron, August, 1968). The present report documents the dose–response of a polycation, diethylaminoethyl-dextran (DEAE-D), on the function of the polynucleotide, I:C, and presents the results of experiments designed to clarify the mechanisms involved.

Materials and Methods. Tissue. Monolayers of human muscle–skin diploid fibroblasts (MSF), in generation 10 to 30, were seeded in 35-mm Falcon tissue culture dishes, and incubated at 37° for 48–72 hr prior to use.

Media. MSF were grown in Leibovitz medium supplemented with 20% fetal calf serum (FCS), 0.03% glutamine, 0.09% arginine, 0.01% glucose, 150 μg/ml of potassium penicillin G and 250 μg/ml of streptomycin sulfate. Similar medium, lacking streptomycin, was used with 2% FCS for maintenance, and without serum for diluting the virus suspensions. I:C was diluted in phosphate buffered saline (PBS) containing 0.01% $CaCl_2$, 0.005% $MgCl_2$ and 150 μg/ml of penicillin.

Additions. The following reagents were used: Poly I:Poly C (Microbiological Associates, Bethesda, Md.) 1 mg/ml, DEAE-D (mol wt ca. 2,000,000; Pharmacia, Uppsala, Sweden), and pancreatic ribonuclease A, 300 units /mg (Worthington Biochemical Corp., Freehold, N.J.).

Virus. The Indiana serotype of vesicular stomatitis virus (VSV) was used for challenge. Its source and storage have been described (4).

Induction of resistance to virus and production of interferon. Monolayers were washed twice with PBS, treated with 2.0 ml of a dilution of I:C or PBS, alone, and then incubated for three hr at 37°. At that time the cells were washed 3 times with 2.5 ml of maintenance medium and incubated with 2.0 ml of the same medium for a further 18 hr at 37°. The medium was then harvested and stored at 4° for subsequent interferon assay. The monolayers were washed twice with 2.0 ml of dilutent and challenged with 0.5 ml of a dilution of VSV containing approximately 100 plaque-forming units (PFU). After incubation for 1 hr at 37° and 2 washes with 2.0 ml of diluent, the monolayers were overlayed with 3 ml of medium containing 0.9% Bacto-Noble special agar, 0.01% $NaHCO_3$ and the ingredients of the maintenance medium. After incubation for 24 hr at 37°, each monolayer received a further 1 ml of similar overlay containing 0.025% neutral red. Plaques were counted 24–48 hr later. Protection against virus infection was measured as the percentage reduction of PFU in the test monolayer when compared to the controls that had been treated similarly but with the PBS lacking I:C.

Interferon assay. Samples were brought to

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room temperature and interferon was determined by a previously described microassay (5). One unit of interferon reduced the cytopathic effect of challenge virus to 50% of that in interferon-free controls.

Toxicity studies. Suspensions of MSF were seeded into tissue culture tubes with threefold serial dilutions of DEAE-D (from 0.36–270 μg/ml) and incubated on a stationary rack at 37°. Observations were made of the attachment and growth of cells and the integrity of the monolayers formed.

Results. Dose response. A 3-hr exposure of monolayers to increasing concentrations of I:C increased the protection of these monolayers against virus challenge 18 hr later. When DEAE-D was present simultaneously during the exposure period there was an enhancement of the protective effect of I:C that varied with the concentration of DEAE-D. In Fig. 1, protection, as measured by the percentage reduction in PFU of the challenge virus in test monolayers compared to identically treated controls lacking I:C, is plotted against $\log_{10}$ of the concentration of DEAE-D. Each line was determined by the values for a single concentration of I:C. In the absence of DEAE-D, the lines would approach the ordinate and show the increasing protective effect of increasing concentrations of I:C. In the presence of increasing concentration of DEAE-D, each curve bends upward as it moves to the right, indicating enhancement of the protective effect afforded by I:C alone.

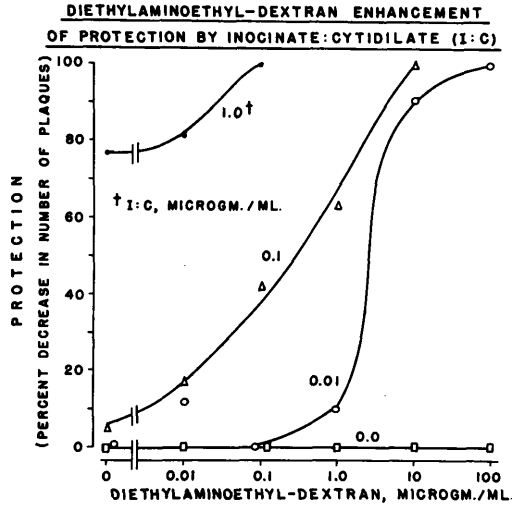

FIG. 1. Exposure of monolayers to various concentrations of I:C with, and without, increasing concentrations of DEAE-D for 3 hr; challenge with VSV 18 hr later.

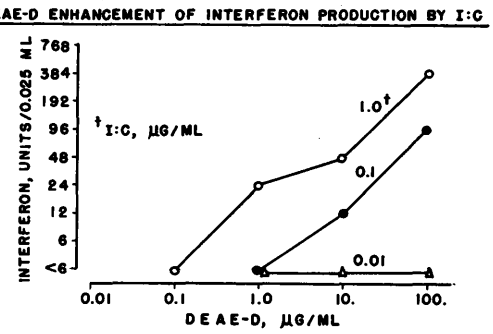

FIG. 2. Exposure of monolayers to various concentrations of I:C with, and without, increasing concentrations of DEAE-D for 3 hr; interferon assayed in the media harvested 18 hr later.

Interferon was detectable in the medium only when the concentrations of I:C and DEAE-D used had been adequate also to provide 100% protection of the cells against direct virus challenge. In Fig. 2, interferon titers of medium removed 18 hr after cells had been exposed to I:C are plotted against the log of the concentrations of DEAE-D. Each curve represents values for a single concentration of I:C, as in Fig. 1. It is evident that induction of interferon production by I:C is also enhanced by DEAE-D and the enhancement again varies with the logarithm of the concentration of DEAE-D.

Observations on mechanism. In the following studies on the mechanism of enhancement by DEAE-D, all experiments were performed in triplicate. The concentrations of I:C and DEAE-D used were chosen on the basis of the results in Figs. 1 and 2, and will be noted.

The rate at which I:C, 1.0 μg/ml, is adsorbed to cells, with and without DEAE-D, was studied by varying the exposure period from 5 sec to 45 min at 37°. The *protection* resulting from the use of 1.0 μg/ml of DEAE-

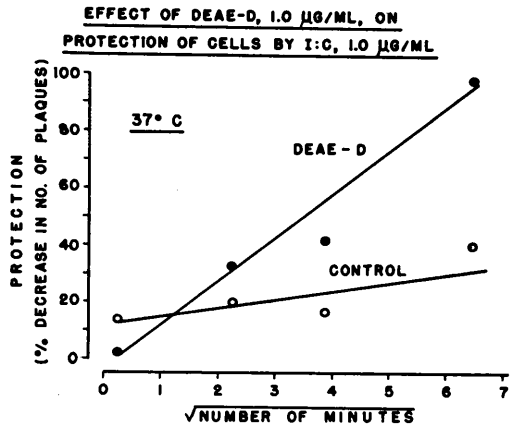

FIG. 3. Exposure of monolayers to I:C alone (control) or to I:C + DEAE-D (DEAE-D) for indicated interval; challenge with VSV 18 hr later.

D is shown in Fig. 3 (where protection is the function measured). The corresponding results, using 10 µg/ml of DEAE-D, are shown in Fig. 4, where *interferon* is monitored. Under the stated conditions, interferon was not detectable in the absence of DEAE-D. Both protection and interferon production by I:C have been plotted against the square root of the number of minutes. It is evident that the relationship is linear with, or without, the presence of DEAE-D. Although the slope of the curve for I:C + DEAE-D in Fig. 3 is greater than that for I:C alone, the lines intersect and the magnitude of the initial points is in the reverse order to that of the slopes.

Since the initial points might well reflect relative rates of attachment, a similar kinetic study was carried out at 4°. After the indicated exposure periods at 4°, monolayers were washed once with 2.5 ml of cold maintenance media at 4° and twice at room temperature with maintenance medium at 37°. The subsequent incubation with maintenance medium and other procedures were carried out as before. The results, using 10 µg/ml of I:C and 1 µg/ml of DEAE-D are shown in

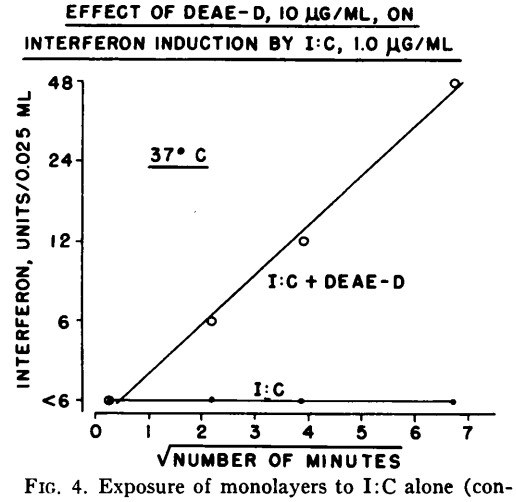

FIG. 4. Exposure of monolayers to I:C alone (control), or to I:C + DEAE-D, for indicated interval; interferon assayed in media harvested 18 hr later.

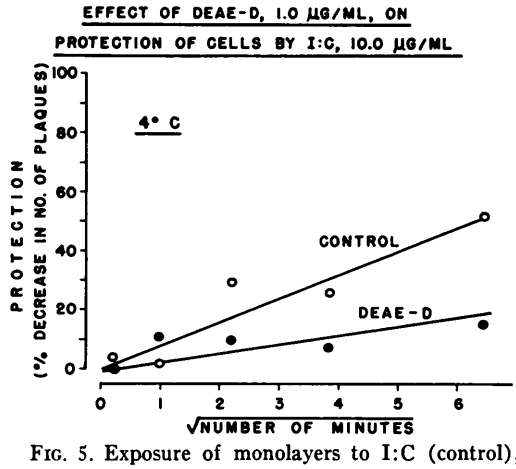

FIG. 5. Exposure of monolayers to I:C (control), or I:C + DEAE-D (DEAE-D), for indicated interval; challenge with VSV 18 hr later.

Fig. 5, where protection is plotted against the square root of the number of minutes. The corresponding results for interferon production, using 100 µg/ml of I:C and 10 µg/ml of DEAE-D are shown in Fig. 6. In each case the slope of the curve of I:C + DEAE-D is less than that of I:C alone. Thus the simultaneous presence of DEAE-D appears to depress the attachment of I:C to cell monolayers under the stated conditions.

To confirm the observation that primarily attachment occurs at 4° under the stated conditions, a similar kinetic study was carried out in which twice as many monolayers were used during the exposure period. Following

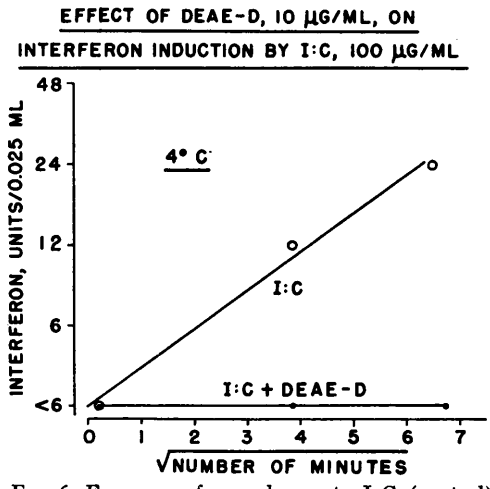

FIG. 6. Exposure of monolayers to I:C (control), or I:C + DEAE-D, for indicated intervals; interferon assayed in media harvested 18 hr later.

the last wash, half of the monolayers were incubated for 60 min at 37° with pancreatic RNase, 100 µg/ml in PBS free of Ca^{2+} and Mg^{2+}, and the other half with similar PBS alone. The monolayers were subsequently washed 3 times with maintenance medium and incubated as before. In Fig. 7, where

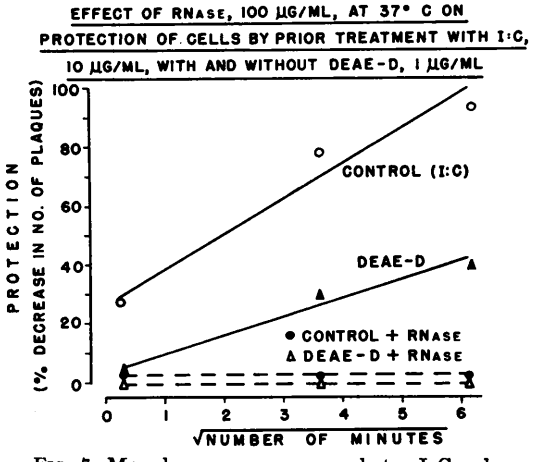

FIG. 7. Monolayers were exposed to I:C, alone (control), or with DEAE-D (DEAE-D), for the indicated time and subsequently exposed to diluent for 60 min at 37°. RNase was used in parallel for the 60-min exposure at 37° of the monolayers after the first exposure to I:C, alone (control + RNase), or with DEAE-D (DEAE-D + RNase).

protection of cells is represented, it is seen that RNase treatment completely negated the exposure of monolayers to I:C, whether or not DEAE-D had been present. Thus, it appears that the I:C associated with cells after incubation at 4° is still susceptible to the action of RNase and under these conditions is presumably in a superficially attached location.

It is noteworthy that the extracellular RNase treatment was not inhibited by the simultaneous presence of DEAE-D. An inhibition of intracellular RNase might be the mechanism by which DEAE-D enhances the biological function of I:C but this was not determined. However, an attempt was made to show such an effect on extracellular RNase. Monolayers were exposed to I:C, 2 µg/ml, for 45 min at 4°, washed 3 times with PBS as before, and then treated for 60 min with RNase, 10 µg/ml, that had been incubated during the previous hour with 1.0, 10.0, or 100 µg DEAE-D/ml at 37°. The monolayers were subsequently washed 3 times and handled as above. In every case the protective effect expected from exposure to I:C at 4° was negated by the subsequent RNase treatment regardless of the concentration of DEAE-D also present.

In the preceding studies, which showed enhancement of I:C activity with DEAE-D, cell monolayers had been routinely exposed to the 2 reagents simultaneously. To determine whether the enhancing effect can also result from treatment of either cells or I:C alone with DEAE-D, prior to exposure of cells to I:C, the following study was made with three sets of monolayers. In set A, cells were treated with DEAE-D, 1 µg/ml, or PBS for 60 min at 37° and then washed 3 times with PBS. This was followed by a 3-hr exposure to I:C, 0.1 µg/ml, 3 washes with maintenance medium, and incubation in the usual way. In set B, the monolayers were treated similarly except that the exposure to DEAE-D occurred simultaneously with the 3-hr exposure to I:C, as in the early studies. In set C, I:C, 10 µg/ml was treated with or without DEAE-D, 1 µg/ml for 60 min at 37°. Dilutions of 1:100, were made to give final con-

TABLE I. Induction of Protection Against Virus Challenge by I:C.
Effect of prior treatment of cells or I:C with DEAE-D and reversal with heparin.

Pretreatment, 1 hr at 37°	Final treatment of cells	Protection[a] (%)
A. Cells in diluent,[b] plus:	I:C, 0.1 μg/ml, plus:	
1. 0	0	0
2. DEAE-D, 1.0 μg/ml	0	42
3. DEAE-D, 1.0 μg/ml	Heparin, 10 μg/ml	12
B. None	I:C, 0.1 μg/ml, plus:	
1. 0	0	0
2. 0	DEAE-D, 1.0 μg/ml	60
3. 0	DEAE-D, 1.0 μg/ml + heparin, 10 μg/ml	7
C. I:C, 10 μg/ml in diluent, plus:	I:C, 0.1 μg/ml in diluent,[c] plus:	
1. 0	0	0
2. DEAE-D, 1.0 μg/ml	DEAE-D, 0.01 μg/ml	5
3. DEAE-D, 1.0 μg/ml	DEAE-D, 0.01 μg/ml + heparin, 10 μg/ml	1

[a] Reduction of PFU of VSV on test monolayers when compared to controls treated similarly but without I:C.

[b] Three washes in PBS after the incubation.

[c] Concentration for final treatment prepared by diluting pretreatment samples 1:100.

centrations of 0.1 μg/ml of I:C but only 0.01 μg/ml of DEAE-D, to treat the cells.

The results are given in Table I. Under the conditions described, there was no protective effect of 0.1 μg/ml of I:C alone as seen in the first line for each of the three sets. In set A, protection, measured as 42% plaque reduction (PR), was found in cells treated with DEAE-D prior to exposure to I:C, but this was less than the protection, 60% PR, resulting from exposure of cells to I:C and DEAE-D simultaneously in set B. When cells were treated with I:C that had been exposed to DEAE-D, and diluted prior to the treatment period, as seen in set C, the protection, 5% PR, was negligible and was consistent with the simultaneous effect of 0.01 μg/ml of DEAE-D still present after dilution.

A similar experiment was done to determine whether interferon production induced by I:C would also be enhanced by pretreatment of cells with DEAE-D. In this experi-

TABLE II. Induction of Interferon by I:C.
Effect of prior treatment of cells with DEAE-D and reversal with heparin.

Pretreatment, 1 hr, 37°	Final treatment of cells	Interferon[a] (units/0.025 ml)
A. Cells in diluent,[b] plus:	I:C, 10 μg/ml, plus:	
1. 0	0	0[c]
2. DEAE-D, 10 μg/ml	0	12
3. DEAE-D, 10 μg/ml	Heparin, 100 μg/ml	0
B. None	I:C, 10 μg/ml, plus:	
1. 0	0	0
2. 0	DEAE-D, 10 μg/ml	96
3. 0	DEAE-D, 10 μg/ml + heparin, 100 μg/ml	0

[a] Assayed in media harvested after 18-hr exposure of cells to I:C.

[b] Pretreatment followed by 3 washes of cells in PBS in each instance.

[c] <6 units/0.025 ml.

ment, sets A and B were the same as before except that the concentration of both I:C and DEAE-D was 10 μg/ml. As shown in Table II, interferon was undetectable after treatment of monolayers with I:C alone. Although interferon was found after pretreatment of cells with DEAE-D followed by I:C, the amount, 12 units/0.025 ml, was considerably less than the 96 units/0.025 ml demonstrated after treatment of cells with DEAE-D and I:C simultaneously.

To determine the degree to which these enhancing effects of DEAE-D were related to its cationic charge, parallel experiments were carried out using the polyanion heparin, in the medium at the time of exposure of cells to I:C. It is evident, from line 3 in each set in both Tables I and II, that enhancement of I:C function by DEAE-D was largely negated by heparin, both when cells had been pretreated with DEAE-D and when cells had been exposed simultaneously to DEAE-D and I:C.

Toxicity. When cells were grown in serial, threefold dilutions of DEAE-D controls without DEAE-D formed homogeneous monolayers after 24 hr, and no difference was observed in concentrations of DEAE-D up to and including 3 μg/ml. At 10 μg of DEAE-D/ml, only a 60% monolayer formed and this was accompanied by some cell degeneration. At still higher concentrations, only an occasional island of degenerated cells and much debris were observed.

Discussion. Field *et al.* (1) have clearly demonstrated that equimolar complexes of certain nucleotide homopolymers can render cells resistant to infection with virus, and can induce the production of interferon. *In vitro*, the susceptibility of most cell strains to this (I:C) type of interferon induction and the molecular weight of the interferon so produced, are similar to those expected with interferon induced by virus, and therefore a similar mechanism of *de novo* production of interferon was first postulated. However, interferon induced by I:C appears more rapidly and is less depressed by inhibitors of RNA and protein synthesis than interferon induced by virus (6).

In vivo, the manner in which interferon is induced by I:C is clearer. The interferon not only appears rapidly and occurs despite inhibition of protein synthesis (7) but also demonstrates cross-tolerance with endotoxin (M. Absher and W. R. Stinebring, personal communication, Feb., 1969), and therefore appears to be a release of preformed interferon, such as follows treatment with endotoxin.

How the complexes induce interferon is still open to question. The resistance to viruses which is conferred directly on cells and not accompanied by detectable interferon, behaves like a low level of interferon. However, the true nature of this resistance, likewise has yet to be established.

The uptake of proteins (8, 9), viruses (4), and particularly of nucleic acids by cells can be enhanced by the presence of polycations. Thus bacterial RNA and DNA have been induced in chick fibroblasts (10); infectious poliovirus RNA was induced in HeLa cells (11), and DNA of infectious simian virus 40 was induced in monkey kidney cells (12). Soon after complexed polynucleotides were found to have these biological functions, it was shown that the processes could be enhanced with DEAE-D (2) and other polycations (Billiau *et al.*, personal communication). The general theories regarding the enhanced uptake of macromolecules by cells in the presence of polycations has been discussed previously (4). The mechanism by which polycations enhance the function of complexed polynucleotides is not known. It has been postulated that the polycations either increase the binding of polynucleotides to cells or protect them from the action of intracellular RNase (personal communications from Billiau *et al.* and from T.C. Merigan, E.D. DeClerq, and G. Bausek, July, 1969).

The present report has documented the dose response of the polycation DEAE-D in enhancing the function of the polynucleotide complex I:C. Using various concentrations of I:C it was shown that both protection of cells against direct virus challenge and production of interferon vary as the logarithm of

the concentration of DEAE-D present during the cell treatment period. However, interferon was detectable only when the cells were completely protected against direct virus challenge. From kinetic studies at 37° it was shown that protection of cells and induction of interferon varied with the square root of the number of minutes of exposure to I:C, whether or not DEAE-D was present. This is the relationship expected if the biological manifestation is a function of the collision frequency of the molecules with the cell surface (13, 14).

The steeper slope of the curve for protection at 37° with I:C plus DEAE-D compared to I:C alone was expected, but the lower initial value for the former was not, therefore the experiment was repeated at 4°. At this temperature the energy-requiring processes of penetration are assumed to be markedly depressed while the physical forces important in attachment should act relatively unimpeded except for the retardation secondary to the increased viscosity (15). At 4° the lesser slope for I:C + DEAE-D was consistent with a depressed attachment rate under these conditions. That only attachment was occurring at 4° was shown by the loss of cell protection when RNase treatment at 37° followed the exposure at 4°. Thus, DEAE-D did not enhance the attachment of I:C to cells but, in fact, depressed it.

If DEAE-D protects I:C from intracellular RNase by binding to the former, one would expect pretreatment of I:C with DEAE-D to be effective in enhancing function, but it was not. It is noteworthy that treatment of cells before exposure to I:C resulted in definite, though less enhancement. This could have been expected if the polycations inactivated intracellular RNase. However, one might also have expected RNase incubated with as much as 100 μg/ml of DEAE-D to lose activity. Although it did not, the question of inactivation of intracellular RNase must remain open because intracellular RNase while within the cell might be inhibited by a substance that has no effect on a different species of RNase in the markedly altered circumstances at the external surface of the plasma membrane.

There is no information as to whether some post-attachment step of penetration is enhanced by DEAE-D. The present findings could be explained if a greater proportion of attached molecules penetrated, *e.g.*, if pinocytoses were enhanced as postulated with respect to the enhancement of protein uptake (16). Alternatively DEAE-D might specifically facilitate the yet to be determined steps in the function of I:C after it is free in the cytoplasm, or possibly in the nucleus.

Summary. In an attempt to clarify the mechanism by which biological effects of the polynucleotide complex, I:C, are enhanced by the polycation, DEAE-D, the dose response of the polycation on cell protection and on the induction of interferon was determined for various concentrations of I:C. Utilizing kinetic studies at 37 and 4° it was possible to show that attachment of I:C to cells is depressed by DEAE-D. In addition, as much as 100 μg/ml of DEAE-D did not inhibit the function of pancreatic RNase in digesting attached I:C. Pretreatment of cells alone before I:C attachment, gave definite, although reduced enhancement of I:C function, and this enhancement could be negated with the polyanion heparin.

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