contributing factor in the pathogenesis of endotoxin shock.

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Ribonucleic Acid of Foot-and-Mouth Disease Virus: An Ultrasensitive Plaque Assay. (31644)

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In 1960(1) the writer carried out a detailed study on the preparation, stability and plating efficiency of ribonucleic acid (RNA) from relatively crude foot-and-mouth disease virus (FMDV). Other workers(2) have recently shown the importance of hypertonic salt solutions for obtaining higher titers of FMDV RNA. While these methods are useful, recent developments indicated that a highly improved procedure might be possible. One such development has been the production of pure FMDV and FMDV RNA(3) possessing well-characterized physical and chemical parameters(4,5). Progress has also been made in the use of additives to improve the plating efficiencies of some viruses and RNAs of the picorna group. For example, Takemoto and Fabisch(6) observed that polycations such as diethylaminoethyl-dextran (DEAE-dextran) enhanced the size and number of plaques of wild-type EMC virus by interfering with the absorption of virus to sulfated polysaccharides present in autoclaved agar. More recently, Pagano and Vaheri(7) obtained a 3- to 4-fold increase in poliovirus plaques in the presence of DEAE-dextran, but did not propose the same mechanism of enhancement as did Takemoto and Fabisch. More significantly, DEAE-dextran increased the sensitivity of the assay of poliovirus RNA by 20- to 100-fold. This enhancement was thought due to a protective action of DEAE-dextran on the RNA and possibly to a direct effect of DEAE-dextran on the host cell.

The present work was begun to increase the sensitivity of the plaque assay of FMDV RNA prepared from purified FMDV. Facilitators of the types described by Dubes and Klinger(8) were ineffective because of limited and non-reproducible enhancements (Bachrach, unpublished results). However, using DEAE-dextran, the increase in sensitivity was even greater than for poliovirus RNA(7). Enhancement of FMDV RNA infectivity ranged from 10^3- to 10^7-fold with good reproducibility in replicate titrations of any given lot of RNA. The number of plaques due to RNA in some experiments exceeded those due to virus by as much as 100-fold. Explanations for these striking results are discussed.

Materials and methods. Virus. Foot-and-mouth disease virus from cattle, type A119, was used after 1 passage in mice, 150 passages in calf-kidney (CK) cultures and one passage in a line of baby hamster kidney cells (BHK).* The BHK cell sheets were grown in rotating 2-liter Baxter bottles in tris (hydroxymethyl)-aminomethane-buffered cell growth medium(3). After infection and harvest, the virus was purified by methods de-

* BHK cells derived from cell line 21, clone 13, were obtained from the American Type Culture Collection Cell Repository, Rockville, Md.

scribed previously(3,4) and stored at 4°C in 0.2 N NaCl, 0.05 M sodium phosphate at pH 7.5. Absorbance-temperature profiles and ultracentrifugal analysis by the synthetic boundary method showed the virus to be undegraded and monodisperse. The concentration of virus was calculated from spectral data using an extinction coefficient, $E^{1\%}_{259\ m\mu}$, of 76.0 (4).

Viral RNA. Infectious RNA was extracted for each run from pure FMDV as follows: Virus was diluted 3-fold in 0.05 M sodium phosphate buffer at pH 7.5 and extracted twice at 4°C with one-half volume of water-saturated phenol containing 0.01% ethylenediaminetetraacetate. The aqueous and phenol phases were separated by centrifugation at 15,000 rpm for 10 minutes in a refrigerated Spinco Model L preparative 40.1 rotor. Phenol was removed from the aqueous phase containing the RNA by 5 extractions with peroxide-free ether. Ether was removed by bubbling nitrogen through the solution.

Infectious RNA was also prepared in the absence of phenol. In this method, the pH of purified virus was adjusted to pH 5.0 with 0.1 M sodium acetate buffer in the presence of 1% sodium dodecylsulfate (SDS)(9). Excess SDS was removed by precipitation in the presence of 0.15 M KCl.

Plaque assays. Standard methods of plaque assay for FMDV and FMDV RNA used in this laboratory have been described(1,11). Calf kidney cultures in 4-oz prescription bottles were used(10). Appropriate dilutions of virus in medium composed of 2% bovine serum, 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution (HLHS medium) are applied in 0.1 ml volumes to predrained cultures. After 90 minutes' incubation at 37°C, the cultures are overlaid with HLHS medium containing 0.02 mg/ml of neutral red and 2% Noble agar (Difco). Infectious RNA is assayed similarly, except that the diluent (HLH medium) lacks serum. In addition, the cultures are triply washed with HLH medium prior to applying RNA, and the time of incubation prior to applying agar overlay medium is 60 minutes. Plaques are counted after 3 days' incubation. Values of plaque-forming units (PFU)/ml in this report are based on the 1:3 dilutions of pure virus which were used for the extraction of infectious RNA. These standard procedures were used for developing the highly sensitive and reproducible assay of FMDV RNA described below. The DEAE-dextran (Pharmacia, Inc.) used was of nominal molecular weight 2 million.

TABLE I. Effect of DEAE-Dextran on Infectivity of Purified FMDV.

Run No.*	Virus: DEAE-dextran in diluent, μg/ml: 0	100	1000	Fractional change†
	log PFU/ml‡			
3, 5	10.4	10.5	—	1.3
7	10.4	—	10.3§	.8
6, 9, 11-15	10.3	—	10.5	1.6
				Avg 1.2

* The same virus preparation stored at 4°C was assayed on different days in primary CK cultures.
† Infectivity with DEAE-dextran/infectivity without DEAE-dextran.
‡ Average for number of runs shown.
§ Prewash and agar overlay also contained 1000 μg/ml of DEAE-dextran.

Absorbance-temperature profiles. The method for obtaining absorbance-temperature profiles of FMDV RNA at 258 mμ has been described(12).

Electron microscope counts. Analytical electron microscopy was carried out essentially as described earlier(13).

Results. Effect of DEAE-dextran on infectivity of purified FMDV. DEAE-dextran at 100 and 1000 μg/ml of HLHS diluent had no significant effect on plaque formation by purified virus (Table I). There was no appreciable effect even when prewash and agar overlay HLHS media contained DEAE-dextran at 1000 μg/ml (Table I, run 7). Assays using HLH diluent were not carried out because serum is required for the maximum plating efficiency of virus(11).

Effect of DEAE-dextran in the inoculum on plating efficiency of FMDV RNA. DEAE-dextran had a striking effect on the plating efficiency of RNA obtained from purified FMDV. In some experiments (Table II) DEAE-dextran was present in the HLH medium used for serially diluting the RNA; in others it was added to each 10-fold dilution

TABLE II. Effect of DEAE-Dextran on Plating Efficiency of FMDV RNA.

Run No.	Virus	Addition	RNA: DEAE-dextran, μg/ml 0	1.6-16	30-50	100-333	500-1000	5000
	log PFU/ml		log PFU/ml					
1	10.7	during dilution	5.4			8.3*		
2	10.7	" "	4.2			7.2		
3†	10.6	during dilution			10.0			
		during & after dilution	5.7					
					10.6			
		after dilution				10.8		
4	10.0	after dilution	4.8		6.8		8.7	
5	10.3	" "	4.7	5.0	6.2	7.9	8.3	
6	10.0	" "	5.1	3.6-4.4	6.2	9.3	12.3	9.5
11	10.1	" "	5.4				8.7	
12	10.4	" "	6.2				8.6	
14‡	10.7	" "	6.3				13.1*	

* RNase at 2 μg/ml prior to addition of DEAE-dextran completely abolished the infectivity of RNA.

† Sodium phosphate at 0.05 M contained 10^{-3} M $MgCl_2$ as diluent for the RNA gave results similar to those shown for HLH diluent.

‡ RNA produced by SDS - pH 5.0 treatment; in all other runs by phenol extraction.

of RNA by means of an additional 2-fold dilution in HLH containing DEAE-dextran. The first method could be complicated by the formation in concentrated solutions of RNA-polycation complexes, containing several strands of each component, which survive further serial dilution. While enhanced sensitivity was obtained by dilution of RNA into DEAE-dextran, better results were obtained by adding DEAE-dextran to serially-diluted RNA (Tables II and IV). The increase in fractional yield (ratio of RNA to original virus) brought about by DEAE-dextran ranged from approximately 10^3 (Table II, runs 1, 2, 11 and 12) to 10^7 (Table II, runs 6 and 14 at 1000 μg DEAE-dextran/ml). Assay of the RNA was extremely sensitive to the amount of DEAE-dextran present (Table II, runs 3-6). In run 5, enhancement was still increasing at 500 μg DEAE-dextran/ml even though this concentration exceeded by about 10 million-fold that (calculated from virion counts) of FMDV RNA molecules in the highest dilution which produced plaques. In run 6, a maximal enhancement was attained at 1000 μg DEAE-dextran/ml; a further increase in the polycation molecules to 5000 μg/ml decreased the effective yield. The reason for the depression of PFU values at 1.6-16.0 μg DEAE-dextran/ml is obscure (Tables II and III, run 6).

Foot-and-mouth disease virus RNA produced by SDS-pH 5.0 treatment (Table II, run 14) was more infectious than the best phenol preparation (Table II, run 6 at 1000 μg DEAE-dextran/ml). In these 2 runs and in run 8 of Table III, the infectivity of the RNA exceeded that of the virus by 10- to 100-fold.

Effect of DEAE-dextran in culture wash fluids and in agar overlay on FMDV RNA infectivity. The effect of DEAE-dextran on cultures was determined (Table III). In run 6, sensitivity of the assay of FMDV RNA, at any level of DEAE-dextran in the RNA inoculum, was not altered when post-absorption culture washes at 60 minutes with HLH medium were used in addition to the standard pre-inoculation washes. DEAE-dextran in pre-inoculation culture washes did not enhance the plating efficiency of FMDV RNA which contained 1000 μg DEAE-dextran/ml (Table III, runs 8 and 13). Inclusion of 1000 μg DEAE-dextran/ml in the agar overlay did not alter this result (Table III, run 8). However, when RNA preparations did not contain DEAE-dextran, prewashes with cationic polymer at 1000 μg/ml enhanced the sensitivity of assay to about the same extent as when

TABLE III. Effect of Treatment of Tissue Cultures with DEAE-Dextran on Their Sensitivity to FMDV RNA.

			DEAE-dextran, μg/ml						
			In RNA†						
Run No.	Virus	In culture washes*	0	1.6	8	40	200	1000	5000
	log PFU/ml		log PFU/ml						
6	10.0	0	5.1	4.4	3.6	6.2	9.3	12.3	9.5
		0‡	5.1	4.3	3.5	6.0	9.3	12.1	9.6
8	10.0	0						11.5	
		0§						11.1	
		1000						11.3	
		1000§						10.9	
13	10.0	0	6.0					8.2	
		100	6.4					8.2	
		1000	8.0					8.1	

* Three pre-inoculation washes with HLH medium at concentration of DEAE-dextran shown plus other culture treatments as specified.
† Added to inoculum after serial 10-fold dilution of the RNA.
‡ Post-incubation washes as well.
§ 1000 μg DEAE-dextran/ml in agar overlay medium.

DEAE-dextran was present in the inoculum only (Table III, run 13).

Sensitivity and reproducibility of FMDV RNA assay in the presence of DEAE-dextran. Table IV shows the assay precision when DEAE-dextran is added either during or after serial dilution of the RNA. Eight replicate titrations were made by each method. The precision of assay was greatest when cationic polymer was added after dilution of the RNA. In addition, the sensitivity for detecting RNA, even though less than that for virus, was significantly higher by this method. In trials where RNA infectivity was equal to or greater than that of virus, reproducible results were also observed (Table II, run 3; Table III, runs 6 and 8). The number of plaques on each of 6 CK cultures for serial 2-fold dilutions of FMDV RNA at 1000 μg DEAE-dextran/ml are shown in Table V. The relationship between plaque numbers and relative concentrations of RNA was approximately linear over a 256-fold change in concentration. In addition, 100 platings of 0.1 ml aliquots of an RNA-DEAE-dextran mixture yielded 19.3 ± 3.8 plaques per culture. The probability of 0.44 for the chance occurrence of the χ^2 value of 16.3 (16 degrees of freedom) indicated that the fit between experimental points and calculated Poisson distribution curve (Fig. 1) was satisfactory.

TABLE IV. Sensitivity and Reproducibility of FMDV RNA Assay with DEAE-Dextran Added by Different Methods.

		RNA	
Run No.	Virus	DEAE-dextran added*	
	log PFU/ml		log PFU/ml†
9	10.4	during dilution	7.8 ± .2
		after "	8.5 ± .1

* Final concentration of DEAE-dextran in inoculum was 1000 μg/ml.
† Ranges at one standard deviation of 8 independent titrations by each method.

Spectral characteristics of FMDV RNA-DEAE-dextran mixtures. A spectral diagram of phenol-prepared FMDV RNA is shown in Fig. 2a. Its $\frac{\text{max}}{\text{min}}$ $\frac{258m\mu}{230m\mu}$ value of 3.1 is characteristic of pure RNA. Fig. 2b presents absorbance-temperature (A-T) profiles of this RNA with and without DEAE-dextran at 1000 μg/ml. The half-melting temperature values (T_m) were 48 and 56°C, respectively. In another trial, the T_m with DEAE-dextran was 55°C. The significance of these melting profiles with regard to the mechanism of protection offered by DEAE-dextran will be discussed.

Discussion. Infectivities of FMDV RNA which are 10 to 100 times greater than those of virus indicate that non-infectious FMDV virions contain infectious RNA cores. This degree of increased infectiousness is possible since non-infectious/infectious virion ratios for purified FMDV range from 83 to 2460

TABLE V. Relationship of Infectivity to Dilution of FMDV RNA in DEAE-Dextran.

Run No.	Culture No.*	Rel. conc. RNA† 1	2	4	8	16	32	64	128	256
		PFU/culture								
11	1	0	0	0	4	7	8	30	41	95
	2	0	1	2	1	5	10	41	50	79
	3	0	1	0	1	4	11	28	38	77
	4	0	0	0	1	8	14	22	36	71
	5	0	0	0	1	6	9	21	39	68
	6	0	0	1	2	9	11	25	40	70
	Avg	0	0.3	0.5	1.7	6.5	10.5	27.8	40.7	76.7

* One-tenth ml of each rel. conc. of RNA plated on 6 CK cultures.
† RNA at 10^{-5} dilution in HLH medium further diluted 2-fold in medium containing 2 mg DEAE-dextran/ml subsequent to serial 2-fold dilutions in medium containing 1 mg DEAE-dextran/ml.

(4). The virus used in the present study contained, by electron microscope count, $10^{13.7}$ virions/ml and an infectivity of $10^{10.0}$ to $10^{10.7}$ PFU/ml. In comparison, extracted FMDV RNA infectivities ranged from $10^{.82}$ to $10^{13.1}$ PFU/ml. The mechanism whereby FMDV infectivity for CK cultures is thus restricted may be related to that which limits and expands the host ranges of picorna viruses and picorna viral RNAs, respectively(14). Thus, specific virus receptors on cells would limit viral infection without inhibiting viral RNA. The reasons, however, why FMDV RNA was most frequently less infectious than virus are obscure. They may involve factors which destroy RNA during its preparation from virus or to variation in the susceptibility of CK cultures. Physical destruction of various amounts of RNA during extraction from FMDV appears to take place as judged from profiles of the RNA product centrifuged on sucrose gradients (Arlinghaus and Bachrach, unpublished results). Explanations for low RNA infectivity which are based on the presence of relatively large amounts of cell-derived ribonuclease (RNase) are less likely since it was found that FMDV RNA infectivity was largely stabilized against otherwise lethal doses of pancreatic RNase by the prior addition of DEAE-dextran. Poor reproducibility which might be expected to occur in cultures of reduced susceptibility was not observed (Table III, run 13; Tables IV and V). Moreover, all lots of primary CK cultures were about equally susceptible to virus.

The mechanism of action of DEAE-dextran appears to be that of complexing with RNA through electrostatic forces. No direct effect *per se* of polycations on the host cell is indicated since DEAE-dextran, unlike the finding for poliovirus(7), did not enhance the infectivity of FMDV. In this regard, the ability of cultures which were prewashed with 1000 μg of DEAE-dextran/ml to enhance the titer of pure viral RNA can best be explained on concentration-dependent reactions near the cell surface which favor RNA-polycation com-

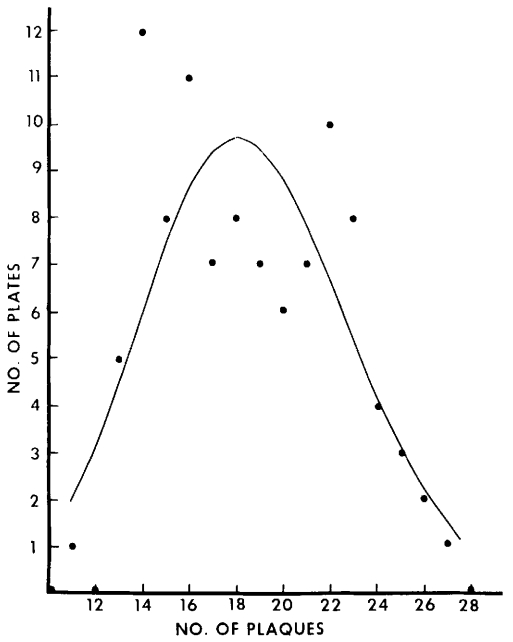

FIG. 1. Frequency distribution of plaques on primary calf-kidney cultures initiated by FMDV RNA containing 1000 μg DEAE-dextran/ml. Points were experimentally determined; curve is calculated Poisson distribution for random distribution of infectious particles.

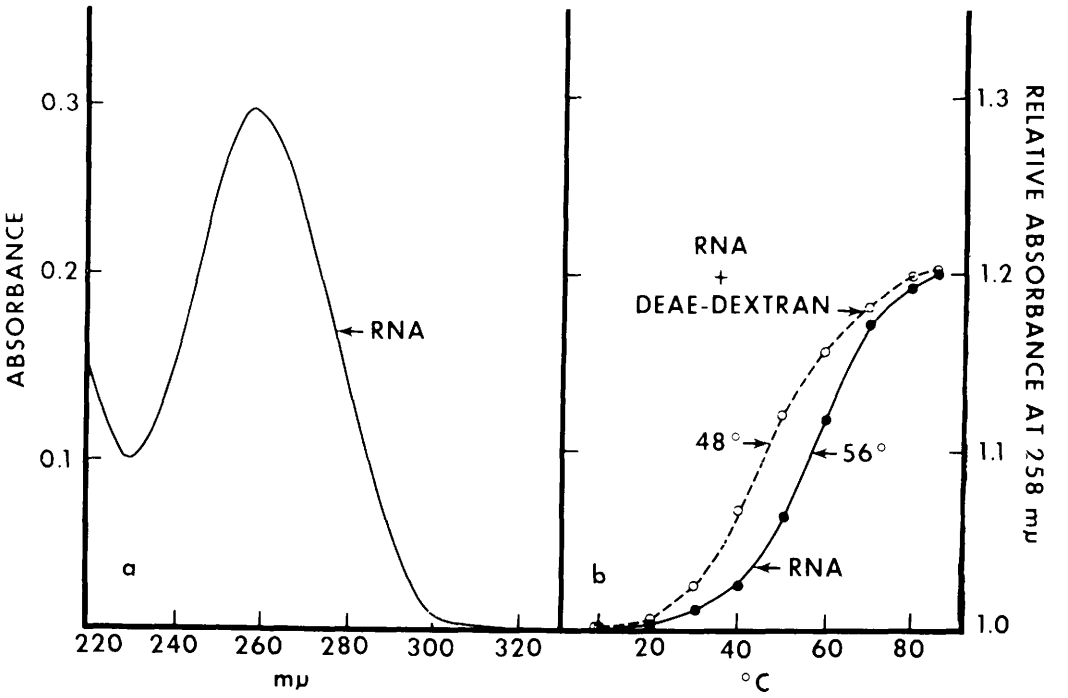

FIG. 2a. Spectral curve for pure FMDV RNA with $max_{258m\mu}/min_{230m\mu}$ absorbance ratio of 3.1. b. Absorbance-temperature profiles for this RNA with and without DEAE-dextran at 1000 μg/ml. Temperatures of half-melting are indicated on the curves.

plexes over RNA-RNase interactions. Protected RNA is not, however, bound to polycations in such a way as to strengthen the secondary and ionic forces which hold FMDV RNA in a helical configuration. Thus, T_m values of RNA-polycation mixtures were slightly lower than that of RNA alone. By comparison, the divalent metallic cation, Mg^{++}, at 10^{-3} M markedly strengthens intramolecular forces in the negatively-charged phosphate-ribose backbone chain of FMDV RNA, raising the T_m by 23°C(12). The decrease in sensitivity which occurred when DEAE-dextran was added during, rather than after, serial dilution of RNA (Table IV) indicates that association between RNA and polycation takes place which is not rapidly reversed. This result is consistent with the formation, in concentrated RNA solutions, of relatively stable RNA-polycation complexes comprised of multiple chains of each component which can be serially diluted further in the presence of DEAE-dextran without rapid dissociation.

Summary. Foot-and-mouth disease virus RNA infectivity for calf-kidney cultures was 10^3- to 10^7-fold higher in the presence of DEAE-dextran than in its absence. By comparison, this polycation had no effect on the sensitivity of the plaque assay of the intact virus. At an optimum concentration of DEAE-dextran of about 1000 μg/ml, the infectivity of different lots of RNA ranged from 10^{-2} to 10^2 times that of the virus. A linear relationship between plaque numbers and dilution was observed, and the assay was highly reproducible. DEAE-dextran did not raise the temperature of half-melting of the RNA.

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Toad Bladder Extract Which Binds Sodium: Role in Sodium Transport.* (31645)

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Leaf and co-workers(1) have found that the urinary bladder of *Bufo marinus* is capable of transporting sodium from the mucosal to the serosal surface against an electrochemical gradient. Susat and Vanatta(2) have reported that there is an ether-soluble compound which binds sodium (ESC-Na) which is extractable from myocardium of dogs. Preliminary experiments in this laboratory showed that sodium was present in ether extracts of the toad urinary bladder.

The hypothesis to be tested by the experiments reported is that if a compound in the extract is involved in sodium transport, then the amount of the compound will vary with some of the factors which affect the rate of sodium transport. Also, if it is involved in sodium transport, it should be present in the bladder which contains a serosal layer and also a mucosal layer of epithelial cells which are involved in the transport mechanisms. On the other hand, little or no compound would be expected to be in only serosal tissue, which does not transport sodium.

Methods. Animals. The urinary bladder of *Bufo marinus* was used in all experiments. The toads were kept in distilled water for at least 20 hours prior to use. Each was pithed and the bladder promptly dissected out for the indicated experiments.

In situ experiments were carried out by cannulating the bladder, and leaving it within the peritoneal cavity of the toad. *In vitro* experiments were carried out by dissecting the bladder free, and removing it with a small piece of gut attached. Each half bladder was then cannulated, and a small piece of the bladder attached to the gut was discarded. The bladders were filled through the cannula with the indicated mucosal solutions, the cannula stoppered, and the bladder checked for leaks. The bladder was then immersed in a beaker in approximately 20 ml of the indicated serosal solution, and incubated for the time indicated at room temperature (20-23°C), unless otherwise indicated. When either sodium-free choline Ringer's solution or potassium solutions were used the bladder was rinsed once with the sodium-free solution. At the end of the experiment a sodium analysis was carried out. There was always sodium present in an amount less that 1 mM/l. This was interpreted as coming from the wall of the bladder, and not due to a leak in the bladder.

Solutions. Regular Ringer's solution contained NaCl 114 mM, $NaHCO_3$ 3 mM, $CaCl_2$ 0.9 mM and KCl 3 mM. Choline Ringer's solution contained choline chloride 114 mM, $KHCO_3$ 3 mM and $CaCl_2$ 0.9 mM. Ringer's solutions containing sodium concentrations of 5, 15 and 40 mM were made by mixing calculated volumes of regular and choline Ringer's solutions. Sodium-free potassium

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