Separation of Plaque-type Variants of Encephalomyocarditis Virus by Chromatography on Calcium Phosphate¹

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Encephalomyocarditis (EMC) virus (K-2 strain) was separated into two distinct peaks of optical density by chromatography on calcium phosphate, eluting at 0.17 and 0.31 M, respectively. The virus in these peaks could not be distinguished by infectivity (plaque-forming units), hemagglutinin activity, electron microscopic appearance, sedimentation coefficient, or base ratios. However, variants producing large plaques predominated in the first peak eluted, whereas variants producing small plaques were concentrated in the second peak. When plaque-purified large and small plaque variants were chromatographed individually, each gave a single peak eluting at 0.16 and at 0.43 M, respectively. However, one small plaque type isolated was eluted in the 0.17 M region expected for large plaque variants, and not with the bulk of the small plaque variants. There were indications that EMC virus (K-2 strain) could be separated into more than two peaks by use of selected elution gradients. The possibility that there may be a relationship between plaque size and point of elution from calcium phosphate is discussed.

Little is known about the structure of animal viruses, owing to the difficulties of producing enough pure virus for study. The K-2 strain of encephalomyocarditis (EMC) virus has been adapted to grow in Krebs-2 mouse ascites tumor cells (7), which, being easy to propagate, presented the opportunity for virus production on a large scale. Therefore, a procedure was sought for purifying EMC virus in large quantities (Burness, *to be published*). One of the techniques investigated was calcium phosphate chromatography, which was introduced for the purification of viruses, including EMC virus, by Taverne, Marshall, and Fulton (20), and which has been used in work leading to the crystallization of EMC virus by Faulkner et al. (4). When the K-2 strain of EMC virus was subjected to chromatography on calcium phosphate, it was noted that virus activity was eluted in two major peaks. Investigation of these peaks has revealed that calcium phosphate chromatography has a capacity to resolve EMC virus variants and that the separation achieved may be related to plaque size.

MATERIALS AND METHODS

Preparation of virus. The EMC virus used in these studies, the K-2 strain derived by Hoskins and Sand-

ers (7), was grown in vitro in continuously agitated suspensions of Krebs-2 ascites tumor cells in Earle's saline (17); radioactively labeled virus was grown in Earle's saline buffered with citrate instead of phosphate and containing 100 μ C of carrier-free ³²P per 10⁷ cells (12). The virus was purified by an acid precipitation-organic solvent-enzyme treatment (Burness, *to be published*). Large and small plaque virus preparations were grown and purified in a similar way, by using as seed virus clones obtained by four successive plaque isolations.

Chromatography on calcium phosphate. Calcium phosphate (brushite) for chromatography was prepared by pumping 0.5 M CaCl₂ and 0.5 M Na₂HPO₄ at 1.7 and 2.6 ml/min, respectively, simultaneously into a 500-ml graduated cylinder already containing 50 ml of water until a total volume of 250 ml was present; during addition, the mixture was slowly stirred magnetically. The calcium phosphate was washed six times by making up to 500 ml with 0.005 M phosphate buffer (pH 7.0), mixing by inverting the cylinder a few times, allowing the calcium phosphate to settle for 5 min, and discarding the supernatant fluid. Experience has shown that calcium phosphate most suitable for chromatography settled to less than the 150-ml mark after standing for 5 min; material which took longer to settle usually gave columns with a slow flow rate. The calcium phosphate could be transferred to a glass column for chromatography at this stage, but it was usually more convenient to store under 0.005 M phosphate buffer in the cylinder at 4 C overnight. The volume of calcium phosphate slurry so produced was about 60 ml, which gave a column of about 20-ml packed volume.

¹ A preliminary account of this work was presented at the Third Federation of European Biochemical Societies Meeting, Warsaw, Poland, 1966.

The columns were prepared by pouring the slurry, diluted with an equal volume of 0.005 M phosphate buffer, into a 2.5-cm diameter Pharmacia glass column cut to 23 cm in length for convenience; the contents of one cylinder were used per column. The calcium phosphate was allowed to settle in the glass column for 15 min, and then 0.005 M phosphate buffer was pumped through at about 3 ml/min for 1 hr or more, until the calcium phosphate surface position was constant. The columns used in the early part of the work were checked at this stage for channeling or skewing of the boundary by washing through a band of bromocresol purple with 0.005 M phosphate buffer. Columns produced in this manner have never channeled, always maintained a fast flow rate, gave good resolution, and were superior to columns produced with calcium phosphate made with fast stirring rates or in larger vessels.

Purified virus was transferred to the column in 10 ml of a mixture of 0.02 M pyrophosphate–0.01 M phosphate buffer (pH 7.8). Crude virus was applied by passing 100 ml of infected cell supernatant fluid through the column. In both cases, after the virus had entered the column bed, it was washed with 80 ml (10 tubes) of 0.005 M phosphate buffer (pH 7.0) before starting the elution gradient.

Elution gradients. The gradients used were prepared by the procedure of Peterson and Sober (15). Eight chambers of a Technicon autograd, made up with 40 ml per chamber of the following solutions, were used. Chambers 1, 2, 4, and 5 contained 0.005 M phosphate (pH 7.0) for gradients A, B, and C. Chambers 3 and 6 contained 0.32 M phosphate (pH 7.0) for gradient A, 0.5 M phosphate for gradient B, and 0.46 M phosphate for gradient C. Chamber 7 contained 0.5 M phosphate for gradients A and B, and 0.46 M phosphate for gradient C. Chamber 8 contained 1.0 M phosphate for gradients A, B, and C.

Gradient A was designed to give a plateau at a phosphate concentration of about 0.12 M, which is suitable for eluting ribonucleic acid (RNA) and most of the possible contaminating proteins (2), followed by a rapid rise in phosphate concentration to elute EMC virus. Gradient B gave a plateau at a phosphate concentration of about 0.18 M, which is just sufficient to begin elution of EMC virus. Gradient C gave a concentration plateau fractionally lower than that produced by gradient B, resulting in better resolution in the 0.18 M region.

The buffers were pumped through the columns at about 3 ml/min, a rate slightly less than that obtained by use of gravity alone. Samples of 8 ml were collected.

Monitoring column eluates. Samples from the column were routinely monitored at 260 m μ , the absorption maximum of EMC virus. The number of hemagglutinin units (HAU) and of plaque-forming units present was measured by conventional methods (11, 17). Radioactivity due to ^{32}P was counted on 1-cm planchets by use of a Philips automatic end-window counter. Phosphate concentration in column eluates was measured by the method of Fiske and SubbaRow (5).

Material for examination in the analytical ultracentrifuge or electron microscope, or for determination of base composition, was obtained after chromatography by combining the contents of peak tubes, centrifuging at 100,000 $\times g$ for 1 hr, and redissolving the virus pellets in 0.1 M phosphate buffer at pH 7.8.

Sedimentation coefficients were determined in a Spinco model E ultracentrifuge fitted with schlieren optics. Electron microscopic examination was performed on negatively stained material (1). Base analysis of the virus RNA was made on virus precipitated with 0.5 N HCl at 0°C then hydrolyzed in 1 N HCl (18). The products, separated by paper chromatography with isopropanol-HCl (22), were located under an ultraviolet lamp, eluted in 0.1 N HCl, and estimated by ultraviolet spectrophotometry.

Protein for Technicon amino acid analysis was prepared from virus by phenol extraction (6) followed by ethyl alcohol precipitation (16), and was hydrolyzed with double glass-distilled 6 N HCl at $108 \pm 1^\circ\text{C}$ for 24, 31, 48, and 72 hr. A 200- μg amount of protein was used for each determination. The figures given in Table 3 represent the average of a determination at each time point, except the figures for threonine and serine which were obtained by extrapolation to zero-time to correct for partial destruction. Valine and isoleucine showed no evidence of incomplete hydrolysis at the early time points, and, therefore, the figures quoted are the average of those obtained for the four time points. Tryptophan is destroyed by acid hydrolysis, and methionine and cysteine were not completely separated so were not included.

RESULTS

Chromatography on calcium phosphate of chemically purified ^{32}P -labeled, stock EMC virus by means of elution with gradient A gave the results shown in Fig. 1. A single peak of hemagglutinin radioactivity, and optical density at 260 m μ started to elute at a phosphate concentration of 0.17 M, and reached a maximum by about 0.34 M. Virtually no label or optical density eluted in the region of 0.11 M or below, as expected for RNA (2), thus demonstrating that the virus had been purified successfully before chromatography. The peak was not always symmetrical, and on occasions showed signs of heterogeneity. This result is similar to that described by Faulkner et al. (4), who used a steep phosphate gradient rising rapidly from 0.1 M upwards, and found a single peak of virus eluting between 0.2 and 0.3 M phosphate concentration. Kaighn, Moscarello, and Fuerst (8) also obtained a single peak containing EMC virus by use of a concave phosphate gradient.

Chromatography with gradient B. Chromatography of stock EMC virus by use of gradient B gave a plateau of phosphate concentration at about 0.18 M (Fig. 2). Two major peaks of both optical density and hemagglutinin activity were eluted, the first at the beginning of the plateau region at a phosphate concentration of 0.17 ± 0.02 M and the second peak at 0.31 ± 0.03 M. At the latter point, the concentration of phosphate in the gradient began to rise rapidly. Rechromatography of the first peak gave a single peak eluting at a phosphate concentration of 0.16 M at its maximum; the second peak had been

FIG. 1. Calcium phosphate chromatography with gradient A (see Materials and Methods) of purified, ^{32}P -labeled, stock EMC virus. Symbols: ●, optical density at 260 $\text{m}\mu$; ×, phosphate molarity; ○, HAU per milliliter; ▲, ^{32}P counts per minute per milliliter.

FIG. 2. Calcium phosphate chromatography with gradient B (see Materials and Methods) of purified stock EMC virus. Symbols: ●, optical density at 260 $\text{m}\mu$; ○, HAU per milliliter; ×, phosphate molarity.

FIG. 3. Rechromatography with gradient B of the first peak obtained from Fig. 2. Symbols: ●, optical density at 260 $\text{m}\mu$; ×, phosphate molarity.

virtually eliminated (Fig. 3). Rechromatography of the second peak gave a broad peak with its maximum eluting at 0.33 M phosphate, and in this instance peak 1 had been almost eliminated (Fig. 4).

Chromatography of ^{32}P -labeled virus with gradient B gave two peaks of radioactivity coincident with optical density and hemagglutinin activity, suggesting that RNA was present in both peaks. This was confirmed by plaque assay, the material in both peaks having similar infectivity. Other features common to the virus in both peaks were that both contained only full particles when examined in an electron microscope, both showed a single peak in an analytical ultracentrifuge, sedimenting with an uncorrected sedimentation coefficient of about 150S, and both contained RNA with a similar base composition (Table 1).

It was considered that the two peaks may be artifacts of the purification procedure used, but crude virus subjected to chromatography with gradient B also gave two peaks of hemagglutinin activity (Fig. 5); thus, the two components were already present in unpurified preparations of virus.

Nature of the two components. Stock EMC virus contains plaque size variants which have been arbitrarily divided into those larger and those smaller than 0.5 μm in diameter; these

FIG. 4. Rechromatography with gradient B of the second peak obtained from Fig. 2.

TABLE 1. *Base composition of EMC virus RNA from variants separated by calcium phosphate chromatography^a*

Base	First peak	Second peak
Guanine.....	23.0	22.6
Adenine.....	25.1	25.0
Cytosine.....	26.0	26.4
Uridine.....	26.0	26.0

^a Figures are the average of three analyses each.

FIG. 5. Chromatography with gradient B of unpurified virus. Symbols: ○, HAU per milliliter; ×, phosphate molarity.

variants are called large and small plaque variants, respectively. The ratio of large plaque variants to small plaque variants present in the preparation before chromatography and in the 0.17 and 0.31 M peaks were compared, and it was found that the ratio was higher in the first peak and lower in the second peak, compared with the original preparation (Table 2). This was the first difference noted between the material in the two peaks. It appeared that chromatography on calcium phosphate showed a tendency to separate particles derived from the K-2 strain of EMC virus into at least two groups by a procedure related to plaque size.

This was investigated further by chromatography on calcium phosphate of plaque-purified large and small plaque variants. The large plaque variant, LPP, gave a single peak of optical density and hemagglutinin activity with gradient B (Fig. 6). The maximum was at phosphate molarity 0.16 ± 0.01 M, which is similar to the position of elution of the first peak on chromatography of stock EMC virus (Fig. 2). Chromatography of the small plaque variant, SPP, under the same conditions gave a single peak eluting at 0.43 M (Fig. 7); although this was a higher phosphate concentration than the maximum for the elution of the second peak of stock EMC virus, it was not higher than the phosphate concentration in the tail fractions of this broad peak. This demonstrated that plaque size variants could be

separated by chromatography on calcium phosphate.

Amino acid analyses of the protein obtained by phenol extraction of the large (LPP) and of the small (SPP) plaque variants revealed that both proteins contained very similar quantities of most of the amino acids present (Table 3). The amount of cysteine or methionine present

TABLE 2. *Effect of chromatography on the ratio of the numbers of large to small plaque variants in EMC virus preparations*

Prepn	No. of large plaques/ no. of small plaques
Original preparation.....	1.6
After chromatography, 0.17 M peak.....	3.3
After chromatography, 0.31 M peak.....	0.6

FIG. 6. Chromatography with gradient B of a large plaque variant of EMC virus. Symbols: ●, optical density at 260 mμ; ○, HAU per milliliter; ×, phosphate molarity.

FIG. 7. Chromatography with gradient B of a small plaque variant of EMC virus. Symbols: ●, optical density at 260 mμ; ○, HAU per milliliter; ×, phosphate molarity.

TABLE 3. Comparison of the amino acid composition of a large plaque (LPP) and small plaque (SPP) variant of EMC virus

Amino acid	Moles amino acid per 100 moles of amino acid recovered	
	SPP	LPP
Asparagine.....	10.3	10.2
Threonine.....	9.3	9.4
Serine.....	7.9	7.8
Glutamine.....	8.8	8.8
Proline.....	5.9	6.5
Glycine.....	7.9	7.7
Alanine.....	8.2	8.2
Valine.....	8.3	8.4
Isoleucine.....	4.1	4.1
Leucine.....	8.7	8.6
Tyrosine.....	2.1	3.1
Phenylalanine.....	6.3	6.0
Lysine.....	5.5	4.8
Histidine.....	1.6	2.4
Arginine.....	4.1	3.8

was not recorded, since these amino acids would have been eluted at the same place under the conditions used. Also, the amount of material eluting in this region was small, making a reliable determination difficult, but suggesting that both amino acids were present in comparatively small amounts, if at all. Some differences were noted in the amino acid contents of these proteins, however, particularly in the amounts of proline, tyrosine, lysine, and histidine present. It is possible that these differences were responsible for the dissimilarities in plaque size produced by the variants and in their chromatographic behavior on calcium phosphate, since both properties are probably mediated through the protein coat. Moscarello and Kaighn (13) have also reported differences in the amino acid composition of plaque size mutants.

Two small plaque variants with different properties. Although chromatography on calcium phosphate resulted in concentration of small plaque variants in the peak eluting at a phosphate concentration of 0.31 M, some were present in the 0.17 M peak (Table 2). It was decided to investigate whether such small plaque variants represented the foot of the 0.31 M peak or whether they have properties different from those of the majority of small plaque types, thus causing their elution in the first peak. Small plaques were isolated from plating of material eluting in the first peak, and without further purification were used as seed to grow a virus pool. Chromatography of two such small plaque pools gave a single, late-eluting peak like the small plaque

variants described earlier. However, one small plaque variant, 28SPb, on chromatography continued to be eluted with gradient B by 0.17 M phosphate, and yet on plating it yielded only small plaques. Chromatography of a mixture of this small plaque variant with the small plaque variant, SPP, already investigated (Fig. 7) gave two peaks of hemagglutinin activity (Fig. 8), thus proving that, although both variants produced small plaques, they contained differences which were demonstrable by chromatography on calcium phosphate.

Takemoto and Liebhaver (19) have shown that the agar normally used for plaque assay contains a sulfated polysaccharide which inhibits the adsorption of certain variants of EMC virus by the cells, thus giving rise to small plaques; large plaque variants are apparently not affected except at much higher concentrations. It was also shown that the inhibitor reduced the hemagglutinin activity of the small but not the large plaque variants; dextran sulfate had similar inhibitory properties (10). The hemagglutinin inhibitory effect of different concentrations of sodium dextran sulfate 2000 (NaDS 2000) was tested on four variants of known elution properties from calcium phosphate. Three variants were known to be eluted by 0.17 M phosphate on chromatography, one being the small plaque variant 28SPb, one the large plaque variant LPP, and the third being a large plaque variant 45LP which was originally isolated from the 0.34 M peak but subsequently was shown to elute in the 0.17 M phosphate peak on rechromatography. The fourth variant investigated was the small plaque variant, SPP, which eluted from calcium phosphate with a peak at a phosphate concen-

FIG. 8. Chromatography with gradient B of a mixture of two small plaque variants.

FIG. 9. Effect of different concentrations of NaDS 2000 on the hemagglutinin activity of EMC virus variants. Symbols: $\circ$, small plaque variant, SPP; $\times$, large plaque variant, LPP; $\bullet$, small plaque and large plaque variants, SP28b and 45LP, respectively.

tration of 0.43 M. The results in Fig. 9 demonstrated that the hemagglutinin activity of the variants eluted in the 0.17 M peak was more resistant to NaDS 2000 than that of variants in a late eluting peak. Although variant 28SPb produces small plaques on plating in agar, it behaves differently from the small plaque variant SPP, and is more like the large plaque variants in its response to NaDS 2000 and its position of elution from calcium phosphate.

Effect of NaDS 2000 on the chromatographic profile. The small plaque variant, SPP, presumably adsorbs NaDS 2000 more readily and is more firmly adsorbed to calcium phosphate than the variants LPP, 45LP, and 28SPb. It was considered that these properties might be related, and it was, therefore, decided to test the effect on the chromatographic profile of having NaDS 2000 in the eluting buffers. A purified, stock EMC virus preparation, supplemented by addition of an equal amount of large plaque variant, LPP, was divided into two parts. One part was subjected to chromatography on calcium phosphate by use of gradient B in the conventional way to confirm that the virus was eluted in two peaks (Fig. 10), although the first peak was larger than normal (Fig. 2). The other part was subjected to chromatography by use of a column which had been prewashed with 0.005 M phosphate buffer containing 0.4 μg of NaDS 2000 per ml and eluting with gradient B containing the same concentration of NaDS 2000. The optical density of the first peak eluted was

FIG. 10. Effect of NaDS 2000 in the eluting buffers on the chromatographic profile of EMC virus. Symbols: $\circ$, elution with 0.4 μg of NaDS 2000 per ml of gradient B; $\bullet$, elution gradient B without NaDS 2000.

lower than that of the control, and the second peak was almost obscured by material eluting much later than normal (Fig. 10). The contents of tubes 40 to 55 (Fig. 10), containing the whole of the late eluting material, were combined, centrifuged at $100,000 \times g$ for 1 hr, redissolved in 0.02 M phosphate buffer (pH 7.8), and rechromatographed by use of gradient B containing no NaDS 2000. Two peaks were obtained (Fig. 11) in positions expected for the conventional first and second peaks. Thus, it appeared that the presence of NaDS 2000 in the eluting buffers caused material normally eluting in the 0.17 M peak to elute later from calcium phosphate, but that this effect was reversible.

Further resolution on calcium phosphate. The presence of NaDS 2000 in the eluting buffers demonstrated that the 0.17 M peak eluted by the conventional gradient B was heterogeneous. This peak was also shown to be heterogeneous by using gradient C, which gives a plateau region

FIG. 11. Rechromatography with gradient B without NaDS 2000 of material eluted late in the presence of NaDS 2000 (tubes 40 to 55, Fig. 10).

slightly below the phosphate concentration given by gradient B. A small amount of the 0.17 M peak obtained by chromatography of ^{32}P -labeled stock EMC virus with gradient B was mixed with a large amount of nonlabeled 0.31 M peak from cold virus and was rechromatographed with gradient C. The points of elution of the 0.17 and 0.31 M material were followed by radioactivity and by optical density, respectively. One peak of optical density but two peaks of radioactivity were eluted. A mixture of a large amount of nonlabeled 0.17 M peak and a small amount of labeled 0.31 M peak similarly gave two peaks of optical density and one of radioactivity on chromatography with gradient C (Fig. 12). This showed that the 0.17 M peak contained at least two components, but whether the second component revealed by gradient C and that revealed by NaDS 2000 were related is not known.

The 0.31 M peak eluted by gradient B also appeared to be heterogeneous, often having an asymmetrical outline (Fig. 4) and occasionally showing evidence for separation into two components. For instance, the profile in Fig. 13 demonstrates rechromatography of a 0.31 M

FIG. 12. Chromatography of mixture of nonlabeled 0.17 M peak and ^{32}P -labeled 0.31 M peak with gradient C. Symbols: ●, optical density at 260 $m\mu$; △, ^{32}P counts per minute per milliliter.

FIG. 13. Rechromatography of a 0.31 M peak. Symbols: ●, optical density at 260 $m\mu$; ○, HAU per milliliter; ▲, ^{32}P counts per minute per milliliter.

eluted peak under conditions thought to be identical with those for Fig. 2, but, where a shoulder is seen in the optical-density profile, the hemagglutinin activity and radioactivity show evidence of separation of the peak into two. Thus, it would appear that stock EMC derived from the K-2 strain contains at least four components which can be separated on calcium phosphate.

DISCUSSION

Only one peak of viral activity had been detected previously when EMC virus (4, 8) and related mengovirus (9) were subjected to chromatography on calcium phosphate by use of simple phosphate gradients. The main difference between our investigation and the previous work was the use of a complex phosphate gradient which has a plateau region just at the phosphate concentration required to elute the 0.17 M eluting variants, but not sufficiently concentrated to elute the late-eluting variants. No other factors are thought to be involved, since it was possible to repeat the results of previous workers by using gradient A which gave a linear phosphate gradient instead of a plateau at the concentration causing elution of virus. Mutants of poliovirus (14) and echovirus 3 have been shown to have different elution characteristics, and herpesvirus (21) yielded more than one peak of virus activity when subjected to chromatography on calcium phosphate, but the separation achieved was not as clear as that given by gradient B with EMC virus.

The main interest of this work is the possible relationship between plaque size and point of elution from calcium phosphate. Plaque size is thought to be influenced by the presence, in the agar used for plating, of a sulfated polysaccharide which preferentially binds the small plaque variants but not the large plaque variants, possibly because of differences in the two viral proteins (10), and, therefore, possibly because of differences in amino acid sequence. It is also highly likely that adsorption to and elution from calcium phosphate involves a unique amino acid sequence in the virus surface protein. Thus, the small plaque variant appeared to have a protein coat which was more firmly bound both by sulfated polysaccharide and by calcium phosphate than the large plaque variant. The possibility existed that a single amino acid sequence controlled both adsorption phenomena. This hypothesis received a setback on isolation of the small plaque variant 28SPb which was not so strongly adsorbed to calcium phosphate as was the small plaque variants in stock EMC or the variant, SPP. However, the response of 28SPb to inhibition by NaDS 2000 was more like that

of a large plaque variant, and a modified hypothesis was considered, that the common amino acid sequence controlled adsorption to calcium phosphate and to sulfated polysaccharide. Variant 28SPb would presumably contain the same sequence present in the large plaque variants, which is responsible for weak adsorption by calcium phosphate and indifference to NaDS 2000, but its properties of forming small plaques would be related to an entirely different amino acid sequence from that causing a production of small plaques by the variants normally encountered.

If the hypothetical amino acid sequences controlling adsorption to calcium phosphate and to sulfated polysaccharide were common, it was reasoned that the presence of NaDS 2000 in the eluant for chromatography might even prevent adsorption of the small plaque variants. Since inhibition by NaDS 2000 depends upon the ratio of virus to inhibitor concentrations (10), it was difficult to decide upon the concentration of inhibitor required in a mobile phase for column chromatography. The concentration decided upon was that known to cause severe inhibition of hemagglutinin activity of the small plaque variants without apparently having any effect on the large plaque variants (Fig. 9). It was, therefore, quite unexpected to find that it was the position of the large plaque, and not the small plaque, variants which was affected by NaDS 2000 (Fig. 10 and 11). It would appear, therefore, that if plaque size and position of elution from calcium phosphate are related, more than a common sequence of amino acids controlling these adsorption phenomena is involved.

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