ADSORPTION PATTERNS OF ENTEROVIRUS RIBONUCLEIC ACID

I. METHODS OF PREPARATION OF CALCIUM PHOSPHATE

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INTRODUCTION

In a number of studies, viral RNA has been subjected to chromatography—in other words, its adsorption properties have been investigated. Methyl albumin columns were used for this purpose[1,2]. The RNA of foot-and-mouth disease virus has been fractionated on calcium phosphate[3]. The present study has been stimulated by the work of BROWN *et al.*[3]; and by the contradictory results of VIZOSO AND BURNESS[4,5], on the one hand, and BERNARDI[6] on the other. Our preliminary findings on the behaviour of RNA on calcium phosphate columns indicated that careful attention must be paid to the preparation of the calcium phosphate: it appears that there is a dependence between the method of calcium phosphate preparation and the adsorption capacity of the RNA. Accordingly, this part of the study is devoted to the method of preparing most adequate calcium phosphate.

EXPERIMENTAL

Virus

Type 1 poliovirus, strain Brunhilde, was used. The viral suspension for RNA extraction was prepared as follows: virus from a single plaque was propagated in a test tube culture of cells, and after their destruction was transferred to a flask culture of cells. The virus was then employed without any further treatment.

Cells

First passage monkey kidney cells were used.

Solutions and media

Reduced medium M 199[7] supplemented with 2% calf serum and 1% La_6 fraction (dried skim milk[8]) was used for cell cultivation. For cells infected with virus or RNA, Earle's balanced salt solution with 0.3% lactalbumin hydrolysate and 1% La_6 fraction was employed.

Cells were washed with buffered salt solution.

The solution used for RNA dilution and its adsorption on to cells consisted of 0.71 *M* NaCl and 0.02 *M* phosphate buffer; its final pH was 7.2.

Extraction of RNA

One part of 80 % phenol was added to one part of virus and stirred for 4 min. The phenol then was separated by centrifugation at 5000 r.p.m. for 5 min. The procedure was repeated once. An equal volume of ether was added to the RNA extract and this was stirred vigorously for 1 min. Ether was then decanted. The procedure was repeated twice. Residual ether was removed by bubbling with nitrogen gas. The whole operation was performed at 4°.

The infectivity of all RNA extracts and eluates was determined by the destruction of cells in tube tissue cultures. Titres were calculated by means of the REED AND MUENCH formula[9].

Calcium phosphate

$CaHPO_4 \cdot 2\ H_2O$ was prepared according to TISELIUS *et al.*[10]. Before mixing, either one or both of the substances from which calcium phosphate is prepared were pretreated by evaporation. Using either the pretreated Na_2HPO_4 or $CaCl_2$, or both, the preparation was continued by TISELIUS' method. Calcium phosphate was always prepared under sterile conditions and stored in 0.001 *M* buffer at 4°.

Evaporation

Each of the substances used in the preparation of the calcium phosphate was dissolved in water and the solution evaporated in a thin layer on a sand bath (110–115°). After rapid cooling the necessary amount of the particular substance was weighed and dissolved in a suitable volume of water.

Chromatography

1 ml of RNA was put on the column, 0.5 cm in diameter and 4 cm in length. Elution was carried out with phosphate buffer solution, pH 7.2, and non-linearily increasing molarity. One ml fractions were collected.

TABLE I

TYPES OF CALCIUM PHOSPHATE

Type	*$CaCl_2$*	*Na_2HPO_4*	*Age*	*For elution pattern see*
1	Non-evaporated	Non-evaporated	5 days	Fig. 1
2a	Evaporated	Non-evaporated	3 days	Fig. 2, curve A
2a	Evaporated	Non-evaporated	9 days	Fig. 2, curve B
2b	Evaporated	Non-evaporated	3 days	Fig. 3, curve A*
2b	Evaporated	Non-evaporated	3 days	Fig. 3, curve B**
3	Evaporated	Evaporated	2 days	Fig. 4, curve A
3	Evaporated	Evaporated***	2 days	Fig. 4, curve B
2a	Evaporated	Non-evaporated	7 months	Fig. 5, curve A
3	Evaporated	Evaporated	22 days	Fig. 5, curve B
3	Evaporated	Evaporated	34 days	Fig. 5, curve C

* $CaCl_2$ and Na_2HPO_4 were mixed at a rate of 100–120 drops per minute.
** $CaCl_2$ and Na_2HPO_4 were mixed at a rate of 30 drops per minute.
*** Other lot of Na_2HPO_4.

RESULTS

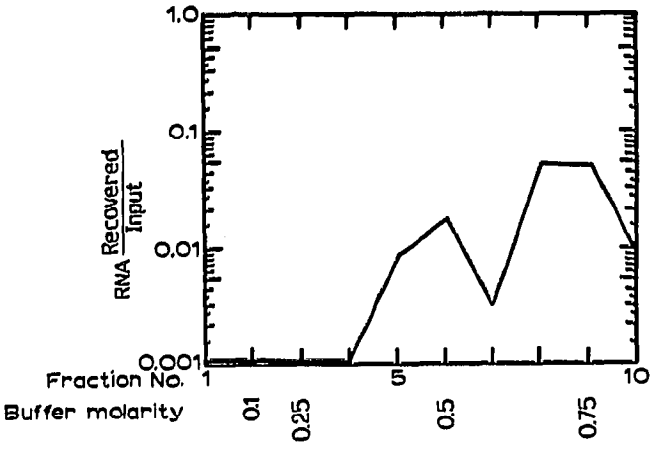
Special attention was paid to the pretreatment of the raw materials from which the calcium phosphate was prepared. Pretreatment is understood to mean the evaporation of either one or both of these substances. A review of the combinations, data on the age of the calcium phosphate and some further data are given in Table I.

Type 1 calcium phosphate

This type of calcium phosphate was prepared from non-evaporated $CaCl_2$ and non-evaporated Na_2HPO_4. Five-day-old preparations were used in the study. The results of elution of poliovirus RNA from such columns are given in Fig. 1. As can be seen, the elution curve had two peaks.

Type 2 calcium phosphate

Type 2a. $CaCl_2$ was dehydrated by evaporation at 110–115°. Otherwise the preparation of calcium phosphate was as before. Three-day (curve A) and nine-day (curve B) gel was used; RNA elution is presented in Fig. 2. In both cases RNA was eluted in two main fractions, but the amount of RNA eluted from the three-day gel was 10 times smaller than that from the nine-day gel.



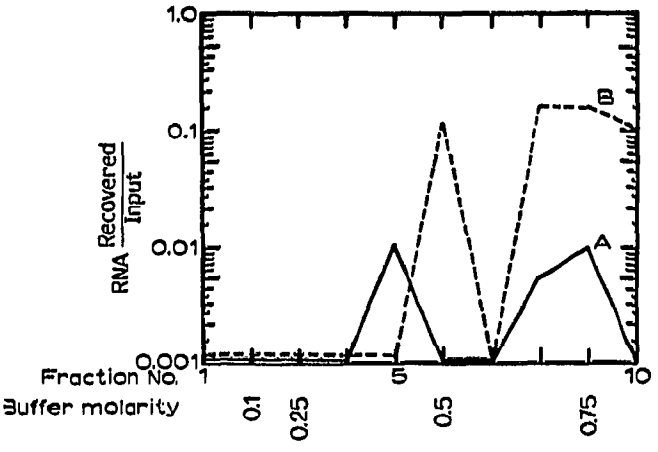


Fig. 1. The elution pattern of infectious poliovirus RNA of type 1 calcium phosphate.

Fig. 2. Type 2a calcium phosphate. The elution pattern of infectious poliovirus RNA of three-day (curve A) and nine-day calcium phosphate (curve B).

Type 2b. Here, the preparation of calcium phosphate was altered in that evaporated $CaCl_2$ and non-evaporated Na_2HPO_4 were mixed at a rate of 100–120 drops per minute, giving what we call "fast" calcium phosphate (curve A). In the second case, the substances were mixed at a rate of 30 drops per min, giving what we call "slow" calcium phosphate (curve B). Fig. 3 presents the results of poliovirus RNA elution from both types of calcium phosphate.

The "fast" calcium phosphate again yielded RNA eluates in two main fractions. In the case of the "slow" calcium phosphate, RNA was also eluted at the same molarities as above, but the clearly defined peaks were missing and, moreover, more than twice as much RNA was eluted.

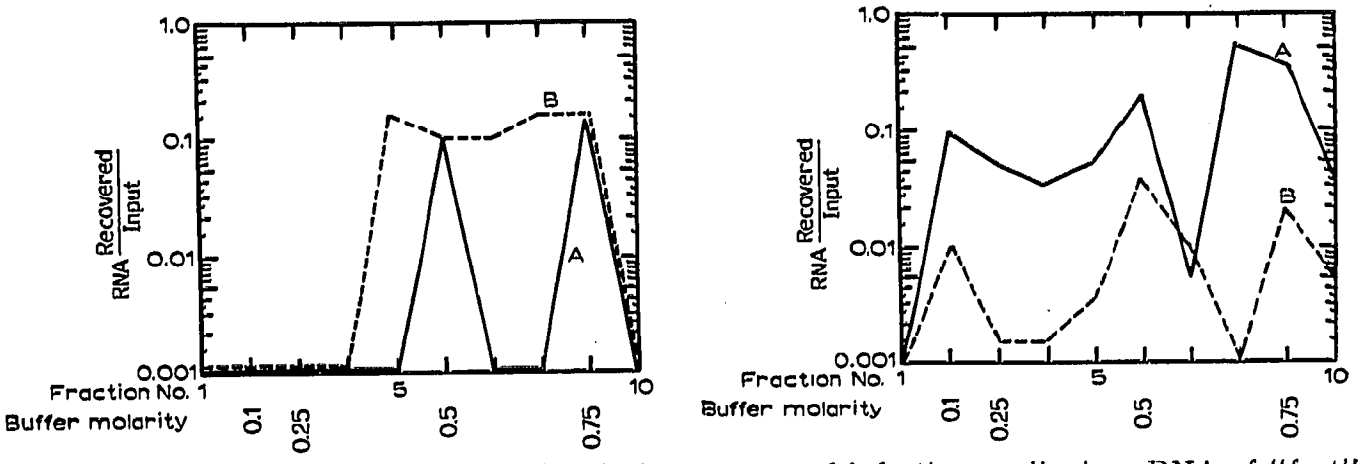


Fig. 3. Type 2b calcium phosphate. The elution pattern of infectious poliovirus RNA of "fast" (curve A) and "slow" calcium phosphate (curve B).

Fig. 4. Type 3 calcium phosphate. The elution pattern of infectious poliovirus RNA of two-day calcium phosphate (curve A). Curve B represents another lot of Na_2HPO_4.

Type 3 calcium phosphate

In this case, both primary substances were evaporated. Fig. 4 gives the pattern of poliovirus RNA elution. RNA was eluted from this type of calcium phosphate in three main fractions (curve A). Two-day calcium phosphate was used. When another lot of Na_2HPO_4 was employed, prepared like the previous one, RNA was also eluted in three main fractions (curve B).

Age of calcium phosphate

After seven months storage, type 2a calcium phosphate, *i.e.* with only $CaCl_2$ dehydrated by evaporation, displayed the same properties as type 3 calcium phosphate. The RNA elution curve from such gel had three peaks (curve A). The adsorption properties of 22-day type 3 calcium phosphate are represented by curve B, while curve C relates to the 34-day type 3 calcium phosphate (see Fig. 5).

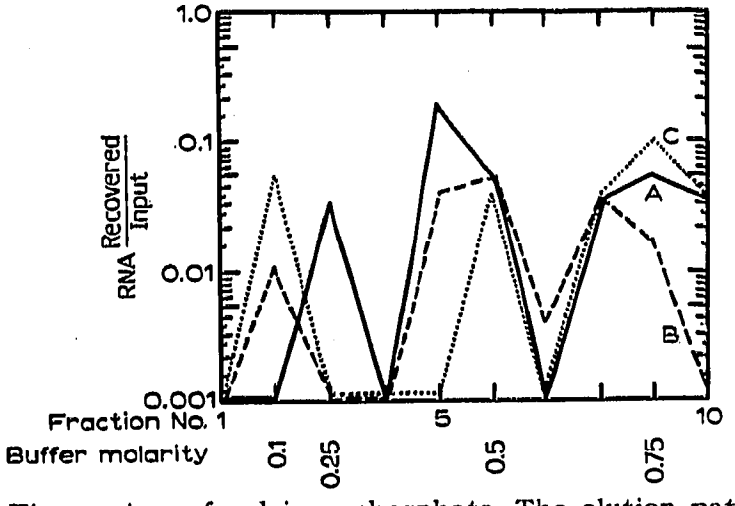


Fig. 5. Age of calcium phosphate. The elution pattern of infectious poliovirus RNA of seven-month-old type 2a calcium phosphate (curve A), of 22-day-old type 3 (curve B) and of 34-day-old type 3 (curve C).

DISCUSSION

The stimulus to the present detailed investigation into the conditions for the preparation of calcium phosphate suitable for reproducible assays on the adsorption properties of infectious enterovirus RNA had come from our preliminary studies which indicated that the question of calcium phosphate preparation demanded careful attention. Furthermore, the difficulties mentioned by MAES[2], who fractionated poliovirus RNA on methyl albumin columns, must be borne in mind. He states: "Although the elution patterns of nucleic acid preparations showed some variability from experiment to experiment, the overall results were sufficiently reproducible to allow a comparison".

The aim of the present study was to achieve the best possible fractionation of infectious enterovirus RNA on calcium phosphate columns; separation into three fractions was considered to be the limit. This could only be achieved if due care was devoted to the preparation of the calcium phosphate. It appeared to be necessary to pretreat, by evaporation, both primary substances from which the calcium phosphate was prepared.

If neither of the substances was evaporated (type 1 calcium phosphate), RNA of poliovirus, strain Brunhilde, was eluted in two fractions. A similar elution pattern was obtained with type 2a calcium phosphate (only one substance was evaporated). The influence of the rate of mixing of $CaCl_2$ and Na_2HPO_4 on the quality of the calcium phosphate is exhibited by the properties of type 2b calcium phosphate. Type 3 calcium phosphate (both components evaporated) gave qualitatively different results. Three main RNA fractions were obtained. Seven months old type 2a calcium phosphate displayed identical properties to type 3 calcium phosphate. The three-fraction pattern on the "old" type 2a calcium phosphate was reliably reproducible—it will depend on personal deliberation which of the two types of calcium phosphate, whether "fresh" type 3 or "old" type 2a is chosen for a particular study.

Sometimes a type 1 calcium phosphate is obtained which can be called "single peak", *i.e.*: poliovirus RNA is eluted from it in one fraction, at buffer molarity 0.75.

Considering all the above data, it appears that poliovirus RNA is essentially eluted with 0.75 *M* phosphate buffer, and two further fractions can only be obtained using lower molarity buffer if the calcium phosphate column is appropriately adjusted. These fractions precede this "basic" fraction. Thus, it is possible to divide infectious poliovirus RNA into three fractions by the procedure described.

SUMMARY

Fractionation of infectious poliovirus RNA on calcium phosphate columns is described. The RNA was extracted from type 1 poliovirus, strain Brunhilde. Three fractions were obtained. A description is given of the methods of preparing calcium phosphate on which such fractionations can be achieved.

REFERENCES

1 H. KUBINSKY AND J. KOCH, *J. Mol. Biol.*, 6 (1963) 106.
2 R. MAES AND E. WECKER, *Z. Naturforsch.*, 19b (1964) 43.
3 F. BROWN, J. F. E. NEWMAN AND D. L. STEWART, *Nature*, 197 (1963) 590.

4 A. D. VIZOSO AND A. T. H. BURNESS, *Biochem. Biophys. Res. Commun.*, 2 (1960) 102.
5 A. T. H. BURNESS AND A. D. VIZOSO, *Biochim. Biophys. Acta*, 49 (1961) 225.
6 G. BERNARDI AND S. N. TIMASHEFF, *Biochem. Biophys. Res. Commun.*, 6 (1961) 58.
7 D. SLONIM, J. MICHL, O. CINNEROVÁ, I. MAREŠ AND M. DŘEVO, *Cesk. Epidemiol. Microbiol. Imunol.*, 9 (1960) 111.
8 J. KOZA AND A. MOTEJLOVÁ, *Cesk. Epidemiol. Microbiol. Imunol.*, 14 (1964) 8.
9 L. J. REED AND H. MUENCH, *Am. J. Hyg.*, 27 (1938) 493.
10 A. TISELIUS, S. HJERTÉN AND Ö. LEVIN, *Arch. Biochem. Biochem. Biophys.*, 65 (1956) 132.