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The Application of DEAE-cellulose Column Chromatography to the Selection of S^+ Revertants and Determination of Reversion Frequency in Populations of S^- Mutants of Fowl Plague Virus

By A. B. GERMANOV, M. I. SOKOLOV AND N. A. PARASIUK
Ivanovsky Institute of Virology, Moscow, USSR

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SUMMARY

A method has been developed for selection of revertants present at low frequency in a population of fowl plague virus. It was based on the differences in elution from DEAE-cellulose columns of the original large-plaque variant (S^+) and polycation-dependent plaque-size mutants (S^-). It was possible to estimate low frequencies of revertants in this way.

INTRODUCTION

The mutants of a number of viruses have been shown to differ in their elution from different adsorbents (Hodes, Zepp & Ainbender, 1960; Roizman & Roane, 1963; Dömök & Simon, 1966). Ion-exchange chromatography has also been used successfully for the separation of certain mutants in genetically heterogenous populations of picornaviruses (Agol *et al.* 1962; Ozaki *et al.* 1965; Maslova & Agol, 1966). Up to the present, column chromatography has not been used for the isolation of rare mutants (or revertants) and for the calculation of their frequency. We have made an attempt to use DEAE-cellulose column chromatography for selection of large-plaque revertants (S^+) from populations of small-plaque mutants (S^-) and for estimation of the frequency of revertants.

METHODS

Viruses. Small-plaque mutants of fowl plague virus (WEYBRIDGE strain) were used. Mutant no. 10 was spontaneous and the others had been induced with the following mutagens: ethyleneimine (no. 118), *N*-nitrosomethylurea (nos. 224 and 231) and *O*-methylhydroxylamine (nos. 411 and 413).

Each mutant stock was prepared by three cycles of plaque purification in chick embryo fibroblast (CEF) monolayers. The size of the plaques formed by these mutants was enhanced in the presence of polycations. When 0·5 mg/ml protamine sulphate was included in the overlay plaque size increased from 1–2 to 3–4 mm diameter in CEF after 5 days incubation at 37 °C. The original S^+ strain and S^+ revertants produced plaques 4 to 6 mm in diameter under the same conditions. They were not influenced by polycations (DEAE-dextran or protamine sulphate).

Treatment with mutagen. In preliminary experiments, the optimal conditions for induction of mutations in fowl plague virus by means of *O*-methylhydroxylamine were determined

(Germanov, Parasiuk & Sokolov, 1971). 4 M-*O*-methylhydroxylamine solution in 0·5 M-phosphate buffer was prepared before use and its pH adjusted to 5·5 with concentrated NaOH solution. Then 0·5 ml of mutagen solution was mixed with 1 ml of 2·6 M-NaCl and 0·5 ml virus suspension, containing 10^6 to 10^7 p.f.u./ml. The mixture was incubated for 1 h at 37 °C. The mutagen was inactivated by dilution (1 : 50) in 0·05 M-phosphate buffer, pH 7·8, containing 1 % acetone. After inactivation of the mutagen 0·2 ml of treated virus suspension was inoculated on to CEF monolayers and incubated for 24 h at 37 °C under liquid overlay (medium 199 without serum). The infective culture fluids were clarified by centrifuging at low speed and loaded onto a DEAE-cellulose column. DEAE-cellulose columns (10 × 1·5) were prepared as has been described by Hydoman (1964).

RESULTS

It has been shown previously that two types of small-plaque (S^-) mutant could be isolated from the original strain of fowl plague virus. The addition of protamine sulphate to the agar overlay enhanced the plaque size of one type of S^- mutant but did not in the case of

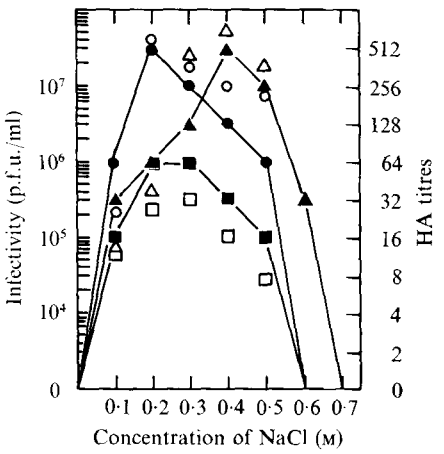

Fig. 1

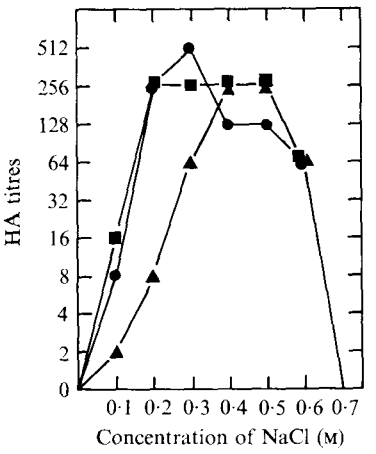

Fig. 2

Fig. 1. Chromatographic pattern of the original S^+ strain and two different S^- mutants. ●—●, HA titres of the original S^+ strain; ▲—▲, HA titres of a polycation-stimulated S^- mutant; ■—■, HA titres of a polycation-insensitive S^- mutant; ○, p.f.u. titres of the original S^+ strain; △, p.f.u. titres of a polycation-stimulated S^- mutant; □, p.f.u. titres of a polycation-insensitive S^- mutant.

Fig. 2. Chromatographic pattern of the original S^+ strain, a polycation-stimulated S^- mutant and an artificial mixture of these viruses. ●—●, HA titres of the original S^+ strain; ▲—▲, HA titres of a polycation-stimulated S^- mutant; ■—■, HA titres of an artificial mixture of these viruses.

the other type (Vorontsova, Germanov & Sokolov, 1971). Subsequently we found that all mutants of polycation-stimulated type, both spontaneous and chemically induced, differed from the original S^+ strain and non-stimulated S^- mutants in elution pattern from DEAE-cellulose columns. The original strain (S^+) and polycation-insensitive mutants (S^-) were eluted mainly by 0·2 to 0·3 M-NaCl solutions while the polycation-stimulated S^- mutants were eluted by solutions of higher ionic strength 0·4–0·5 M (Fig. 1).

The correlation between polycation stimulation of plaque formation and change of

Table 1. *Separation of the original S⁺ virus and polycation-stimulated S⁻ mutant (no. 231) from a mixed population by means of DEAE-cellulose column chromatography*

Concentration of NaCl solutions (M)	Mixed population		S⁻ mutant		Original S⁺ virus	
	Titre (p.f.u./ml)	Ratio S⁺/S⁻	Titre (p.f.u./ml)	Ratio S⁺/S⁻	Titre (p.f.u./ml)	Ratio S⁺/S⁻
0·1	$2·4 \times 10^4$	50/0	$7·6 \times 10^2$	9/160	$2·5 \times 10^4$	55/0
0·2	$1·0 \times 10^6$	20/0	$4·4 \times 10^3$	1/51	$1·3 \times 10^6$	27/0
0·3	$5·4 \times 10^6$	12/1	$4·4 \times 10^5$	0/90	$3·4 \times 10^6$	93/0
0·4	$2·0 \times 10^6$	19/28	$2·1 \times 10^6$	0/44	$1·4 \times 10^6$	31/0
0·5	$2·1 \times 10^6$	5/34	$4·1 \times 10^6$	0/57	$7·7 \times 10^5$	17/0
0·6	$2·7 \times 10^6$	5/159	$2·0 \times 10^6$	0/239	$3·9 \times 10^5$	79/0

Table 2. *The frequency of spontaneous and O-methylhydroxylamine-induced S⁺ revertants in populations of polycation-stimulated S⁻ mutants*

Mutant	Nature of reversion	Expt.	Total virus in 0·2 M-NaCl eluates (p.f.u.)	Ratio $S^+/(S^+ + S^-)$	Total virus in 0·6 M-NaCl eluates (p.f.u.)	Revertant frequency
224	Spontaneous	1	$3·9 \times 10^2$	3/264	$2·2 \times 10^6$	$8·0 \times 10^{-6}$
	Induced	2	$1·1 \times 10^3$	41/72	$9·8 \times 10^5$	$2·5 \times 10^{-3}$
		3	$3·4 \times 10^3$	46/69	$9·4 \times 10^6$	$9·7 \times 10^{-4}$
118	Spontaneous	1	$1·0 \times 10^3$	2/475	$1·2 \times 10^6$	$1·3 \times 10^{-5}$
	Induced	2	$5·2 \times 10^3$	9/231	$1·4 \times 10^6$	$5·6 \times 10^{-4}$
		3	$5·9 \times 10^3$	22/119	$7·0 \times 10^6$	$6·2 \times 10^{-4}$
231	Spontaneous	1	$3·4 \times 10^2$	6/306	$8·2 \times 10^6$	$3·2 \times 10^{-6}$
	Induced	2	$1·0 \times 10^3$	0/71	$1·1 \times 10^7$	$< 4·8 \times 10^{-6}$
		3	$6·8 \times 10^2$	3/681	$5·3 \times 10^6$	$2·2 \times 10^{-6}$
411	Spontaneous	1	$1·1 \times 10^3$	4/463	$1·7 \times 10^6$	$1·1 \times 10^{-5}$
	Induced	2	$1·0 \times 10^3$	2/458	$1·5 \times 10^6$	$1·0 \times 10^{-5}$
		3	$1·1 \times 10^4$	1/206	$4·6 \times 10^7$	$4·4 \times 10^{-6}$
413	Spontaneous	1	$6·2 \times 10^2$	1/129	$5·4 \times 10^6$	$2·0 \times 10^{-6}$
	Induced	2	$3·7 \times 10^2$	1/253	$4·1 \times 10^6$	$1·3 \times 10^{-6}$
10	Spontaneous	1	$6·9 \times 10^2$	2/305	$1·2 \times 10^6$	$1·5 \times 10^{-5}$
	Induced	2	$6·1 \times 10^2$	70/199	$1·0 \times 10^6$	$8·5 \times 10^{-4}$

chromatographic properties allowed us to use column chromatography for the selection of spontaneous and chemically induced S^+ (polycation-insensitive) revertants in population of S^- (polycation-stimulated) mutants. Revertion to the S^+ phenotype was accompanied by a change in chromatographic pattern.

In Expt. 1 the column was loaded with an artificial mixture of equal volumes (50 ml) of the original S^+ strain and stimulated S^- mutant no. 231 (5×10^5 p.f.u. of each). The column was washed free from unadsorbed virus with 50 ml of 0·05 M-phosphate buffer and then virus was eluted by 10 ml of 0·1, 0·2, 0·3, 0·4, 0·5 and 0·6 M-NaCl solutions in 0·05 M-phosphate buffer, pH 7·2. Both the original S^+ strain and the stimulated S^- mutant were loaded onto columns separately as controls. The infectivity, large plaque/small plaque ratio and haemagglutinating (HA) activity were determined in the eluates (Table 1, Fig. 2).

Results presented in Fig. 2 and Table 1 show that most of the infectivity and the HA activity of the original S^+ strain was eluted by 0·2 to 0·4 M-NaCl solutions, while that of the stimulated S^- mutant was eluted by 0·4 to 0·6 M-NaCl solutions. When a mixture of these viruses was eluted, both the infectivity and HA-activity were evenly distributed among the eluates.

The fact of importance was the marked difference in large/small plaque ratio in eluates. It may be seen that column chromatography allowed the separation of large and small plaque-forming virus from an artificial mixture of the original S^+ strain and S^- mutant.

In the eluates of 0·1 and 0·2 M-NaCl solutions small plaques were absent (see column 3 of Table 1). In 0·3 M-NaCl solution large plaques represented the bulk of the population, whereas in 0·4 to 0·6 M-NaCl solutions small plaques were predominant. The controls (see columns 4 and 6 of Table 1) showed that the quantity of stimulated S^- mutant (p.f.u./ml) eluted in 0·1 and 0·2 M-NaCl solutions was 100 to 1000-fold lower than that of the original S^+ virus. Thus it seems possible to use DEAE-cellulose column chromatography for detection of S^+ revertants in populations of polycation-stimulated S^- mutants since the proportion of S^+ plaques would be enriched in 0·2 M-NaCl eluate.

Since the quantity of stimulated S^- mutant eluted in 0·2 M-NaCl solution was approximately 100 to 1000-fold lower than in 0·5 to 0·6 M-NaCl solutions, and the quantity of the original S^+ strain eluted in 0·2 M-NaCl was higher than in 0·6 M-NaCl solutions, we supposed that S^+ revertants in a population of S^- mutants should be enriched more than 1000-fold in 0·2 M-NaCl eluate.

Indeed a small proportion of large plaques was detected in 0·1 and 0·2 M-NaCl eluates of stimulated S^- mutant no. 231 (see column 5 of the table 1). Clones isolated from these plaques behaved as stable revertants indistinguishable from the original strain in serological tests (HI-test).

On the basis of these data the following method was used for the determination of the revertant frequency in populations of polycation-stimulated S^- mutants. The columns were loaded with 50 to 100 ml of culture fluid containing approximately 10^5 p.f.u./ml of the appropriate S^- mutant. First the column was eluted with 10 ml of 0·2 M-NaCl solution to detect the presence of large plaques revertants. Then the rest of the virus was eluted with 20 ml of 0·6 M-NaCl solution. The 0·2 and 0·6 M-NaCl eluates were titrated by plaque technique and the ratio of large plaques to the total number of plaques in eluates was determined.

The following equation was used to calculate the reversion frequency

$$= \frac{F \times S^+/(S^+ + S^-) \times T_1 \times V_1}{T_1 \times V_1 + T_2 \times V_2},$$

where

$S^+/(S^+ + S^-)$ is the ratio of large plaques to total plaques in the 0·2 M-NaCl eluate;
T_1 is the virus titre (p.f.u./ml) in the 0·2 M-NaCl eluate;
V_1 is the volume of the 0·2 M-NaCl eluate;
T_2 is the virus titre (p.f.u./ml) in the 0·6 M-NaCl eluate;
V_2 is the volume of the 0·6 M-NaCl eluate.

As can be seen from the data in Table 1, the original large plaque virus was eluted by 0·2, 0·3 and 0·4 M-NaCl solutions at approximately the same level and by 0·5 M-NaCl solution at a slightly lower level. Therefore in order to calculate the total number of revertants in the eluated virus population, the number of large plaques in the 0·2 M-NaCl eluate, i.e. $S^+/(S^+ + S^-) \times T_1 \times V_1$, should be multiplied by a factor F.

$$F = \frac{E_{0\cdot1} + E_{0\cdot2} \ldots E_{n-0\cdot1} + E_n}{E_{0\cdot1} + E_{0\cdot2}}$$

where,

$E - S^+$ virus titre in NaCl eluate of appropriate concentration;
n = final concentration of NaCl solution.

Table 3. *Determination of the ratio $S^+/(S^+ + S^-)$ in artificial mixtures of the original S^+ strain and a polycation-stimulated S^- mutant (no. 231) by means of DEAE-cellulose column chromatography*

Ratio S^+/S^- prior to chromatography	Total virus in 0·2 M-NaCl eluate (p.f.u.)	Ratio $S^+/(S^+ + S^-)$ in 0·2 M-NaCl eluate	Total virus in 0·6 M-NaCl eluate (p.f.u.)	Experimental values of ratio $S^+/(S^+ + S^-)$
1:1	$7{\cdot}0 \times 10^4$	123/123	$5{\cdot}1 \times 10^5$	$\frac{4 \times 7{\cdot}0 \times 10^4}{7{\cdot}0 \times 10^4 + 5{\cdot}1 \times 10^5} = 0{\cdot}48$
1:5	$1{\cdot}1 \times 10^4$	18/22	$2{\cdot}8 \times 10^5$	$\frac{4 \times 18 \times 1{\cdot}1 \times 10^4}{22(1{\cdot}1 \times 10^4 + 2{\cdot}8 \times 10^5)} = 0{\cdot}12$
1:10	$7{\cdot}1 \times 10^3$	186/229	$1{\cdot}9 \times 10^5$	$\frac{4 \times 186 \times 7{\cdot}1 \times 10^3}{229(7{\cdot}1 \times 10^3 + 1{\cdot}9 \times 10^5)} = 0{\cdot}12$
1:1000	$8{\cdot}4 \times 10^2$	2/27	$2{\cdot}6 \times 10^5$	$\frac{4 \times 2 \times 8{\cdot}4 \times 10^2}{27(8{\cdot}4 \times 10^2 + 2{\cdot}6 \times 10^5)} = 0{\cdot}00095$

A value for F can be obtained as follows using the data table 1 (see column 6), i.e.

$$F = \frac{(0{\cdot}02 + 1{\cdot}3 + 3{\cdot}4 + 1{\cdot}4 + 0{\cdot}8 + 0{\cdot}4) \times 10^6}{(0{\cdot}02 + 1{\cdot}3) \times 10^6} = 5{\cdot}6.$$

Values of F were calculated from the data of three other separate experiments. The values of F were 3·2, 3·4 and 4·1 respectively, thus giving a mean value of 4·07.

Table 2 presents the results of determination of the frequency of S^+ revertants by this method in the case of different S^- mutants of spontaneous or chemically induced origin. The mutants differed in the frequency of spontaneous revertants, but in all cases it was rather low.

Treatment with 1 M-*O*-methylhydroxylamine considerably increased the frequency of revertants in three S^- mutants. These S^- mutants and their S^+ revertants had the same susceptibility to inactivation by the mutagen, therefore selection of S^+ revertants resistant to mutagen was ruled out.

Experiment 2 was performed to evaluate the relation between the value of revertant frequency obtained experimentally by this method and its actual value (Table 3). For this purpose the columns were loaded with an artificial mixture of the original strain and S^- mutant no. 231 in the proportions 1:1, 1:5, 1:10 and 1:1000 respectively.

Table 3 shows that the values for the frequency of large plaques obtained experimentally approximated the actual values. Thus this method allowed not only selection of rare S^+ revertants but also estimation of spontaneous and induced mutability.

DISCUSSION

A method of quantitative estimation of the rate of spontaneous and induced mutations is an essential requirement for the investigation of mutagenic activity and specificity and for the measurement of the genetic stability of virus stocks. Mutagenic activity can be measured by the induction of forward mutants. Induction of reverse mutations (reversions) by appropriate mutagens allows determination of the type of mutation at the molecular level, i.e transition or transversion (Freese & Freese, 1966). However, mutants that have a high rate of spontaneous reversion and non-revertable mutants are not suitable for this purpose. A high level of spontaneous reversion makes it difficult to estimate the efficiency of mutagenic action, and non-revertable mutants may be deletions, multi-site or frame-shift mutations.

The application of partial separation of S^+ and S^- variants by means of DEAE-cellulose chromatography enabled us to determine low frequencies (10^{-6}) of S^+ revertants. This method also allowed us to select mutants that reverted with low frequency for further study of the mutagenic specificity of several chemicals: *O*-methylhydroxylamine, hydroxylamine, nitrous acid, ethyleneimine, ethylmethane sulphonate, *N*-nitrosomethylurea (Germanov, *et al.* 1971). Finally this method can be applied to the detection of rare recombinants in crosses of different S^- mutants.

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