Acta virol. **25** : 205—212, 1981

PURIFICATION AND CONCENTRATION OF RABIES VIRUS BY CHROMATOGRAPHY ON CHEMICALLY MODIFIED POROUS SILICATES

I. V. KRASILNIKOV, T. A. AKSENOVA, B. V. MCHEDLISHVILI

Institute of Polyomyelitis and Viral Encephalitides, U.S.S.R. Academy of Medical Sciences, 142782 Moscow, U.S.S.R.

Received December 21, 1979; revised December 18, 1980

Summary. — Tissue culture rabies virus was purified by gel chromatography on chemically modified macroporous glasses (MPG). Modification of the MPG surface by egg or human albumin prevented rabies virions from interaction with the glass surface so that all purified virus was eluted from the column. Chemically modified MPG could be used on a preparative scale for the purification of both infectious and inactivated rabies virus, the immunogenicity of the latter having been preserved. Protein content of the purified virus fractions was up to 4 μg/ml, of which virus protein represented up to 5%.

Key words: rabies virus; gel chromatography; macroporous glass

Introduction

Chromatography on porous silicates on analytical or preparative scale has been used for the purification of various viruses of vertebrates (Haller, 1967; Bresler *et al.*, 1969, 1974, 1977). Of the two known variants of chromatography on macroporous silicates — gel chromatography and adsorption chromatography — only the former proved to be suitable for rabies virus (Mchedlishvili *et al.*, 1972). The highest yields of purified virus were observed with silicates pretreated with blood serum or polyethers (Hiatt *et al.*, 1971; Mchedlishvili *et al.*, 1972). But the yield of virus from such glasses decreased with time due to elution of the modifying substances. The latter are not eluted if the silicate surface is chemically modified, i.e. when the modifying substances are covalently linked to hydroxyl groups on the silicate surface.

Porous glasses modified in this way were used for gel chromatography of influenza (Mchedlishvili *et al.*, 1976) and tick-borne encephalitis (Krasilnikov *et al.*, 1977) viruses. In the present paper we are reporting the results concerning purification of rabies virus by gel chromatography on chemically modified silicates and demonstrate the possibility to concentrate rabies virus by adsorption chromatography on such carriers.

Materials and Methods

Virus. Fixed rabies virus strain Vnukovo 32 was grown in primary Syrian hamster kidney cell cultures (Aksenova *et al.*, 1966). Earle's solution containing 0.5% lactalbumin hydrolysate and 2% human albumin was used as maintenance medium. Virus-containing material was inactivated by irradiation with ultraviolet light (Morogova *et al.*, 1973).

Porous silicates. Macroporous glasses (MPG) prepared in the Institute of Silicate Chemistry, U.S.S.R. Academy of Sciences (Zhdanov, 1952) had pore diameters from 0.19 to 0.36 μm and a porosity from 1.6 to 2.2 cm^3/g. Silochromes prepared at the M. V. Lomonosov University, Moscow (Bebris *et al.*, 1971) had pore diameters from 0.03 to 0.17 μm and a porosity of 1.2—1.8 cm^3/g. In the present study we used chemically modified MPG and silochromes described by Mchedlishvili *et al.* (1976) and Krasilnikov *et al.* (1977) as characterized in Table 1.

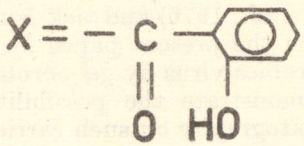
Gel chromatography. Analytical columns 0.8 × 12 cm were filled with the silicates and equilibrated with 0.05 M Tris-HCl buffer containing 0.15 M NaCl, pH 7.7. Thereafter 0.5 ml of virus suspension was placed on the column and elution was done with the same buffer at a rate of 2 cm/min. Fractions of 0.5 ml were collected and assayed for virus and protein contents. Preparative gel chromatography was carried out on 5.6 × 85 cm columns; the volume of the virus samples placed on the column varied from 110 to 480 ml and fractions of 200 ml were collected. The yield of purified virus in gel chromatography experiments was calculated by the formula

$$\% \text{ virus yield} = \left(\sum_{1}^{n} V_f \times T_f / V_c \times T_c\right) \times 100\%$$

where V_c, V_f = volume of the virus suspension examined and of the eluted fractions, respectively, and T_c, T_f = titres of virus in the virus suspension examined and in eluted fraction, respectively; 1, 2, ... n = number of eluted fractions examined.

Table 1. Characteristics of chemically modified MPG and silochromes

Sample No.	Type of silicate	Chemical formula of silicate	Functional groups or protein, mgeq/m^2
1	MPG	—Si—$(CH_2)_3$—NH—X	0.010
2	MPG	—Si—$(CH_2)_3$—NH=CH_2—$(CH_2)_3$—CH_2=NH—egg albumin	0.013
3	MPG	—Si—$(CH_2)_3$—OH	0.008
4	MPG	—Si—$(CH_2)_3$—NH—C(=O)—$(CH_2)_2$—C(=O)—OH, OH	0.011
5	Silochrome	—Si—$(CH_2)_3$—NH_2	0.005
6	MPG	—Si—$(CH_2)_3$—NH=CH_2—$(CH_2)_3$—CH_2=NH—human albumin	0.015
7	MPG	—Si—$(CH_2)_3$—NH=CH_2—$(CH_2)_3$—CH_2=NH—human albumin	0.007
8	Silochrome	—Si—$(CH_2)_3$—OH	0.01



Adsorption chromatography. A 0.8 × 5 cm column was filled with an aqueous suspension of chemically modified MPG or silochrome with a free OH-group of an alcoholic character (Table 1, samples 3 or 8). Eighty ml of virus suspension were passed through the column at a rate of 0.5 cm/min. Virions were adsorbed on to the carrier, the column was "saturated" with virus. After passage of the virus supension, non-adsorbed virus and proteins were washed out from the column with 20 ml of 0.05 M Tris-HCl buffer, pH 7.8 at the same rate. Virus adsorbed on to the carrier was eluted by 0.1 M Tris-HCl buffer, pH 8.6, containing 0.2 M NaCl. Fractions of 2 ml were collected and assayed for virus and protein contents.

Virus and protein assay. Infectivity of the virus was assayed by intracerebral titration in 6—7 g mice; the titres were calculated by the method of Reed and Muench. Activity of inactivated virus was evaluated by the index of immunogenicity which was calculated by the method of the National Institutes of Health, U.S.A. (WHO, 1975). Elution of protein from the columns was monitored by absorbance at 280 nm. Protein contents were estimated by the method of Lowry *et al.* In purified preparations, the proteins were preliminarily concentrated by 10% trichloroacetic acid. The concentration of virus particles was estimated by electron microscopy according to Sharp (1965). Virus protein contents in purified preparations were estimated by the formula $C = \bar{p} \times M$, where C = rabies virus protein concentration in μg/ml; $\bar{p}$ = mean number of virus particles per ml as determined by electron microscopy; and M = mass of proteins in one virus particle equal to 5.5×10^{-10} μg, determined according to Sokol *et al.* (1971).

Results

Purification of rabies virus

The results of analytical experiments on gel chromatography of rabies virus on chemically modified silicates are presented in Table 2. Virus adsorption did not occur only on porous silicates modified by either egg or human albumin. From such carriers the elution of purified virus was complete and, as judged from titres of the purified virus, the virus zone was only insignificantly broadened in the course of gel chromatography. The degree of virus purification from ballast proteins was high. Up to 99.8% of the proteins were removed in the course of gel chromatography, indicating good separation of the viral and protein zone. Such separation in our experiments was due to

Table 2. Purification of rabies virus on chemically modified MPG

MPG sample No. *	Virus titre (log LD_{50}/ml) after purification**	Virus yield %***	Degree of virus purification E_{280} before/after purification	%
1	3.0	0.5	7.2/0.03	99.6
2	5.4	90.0	7.2/0.05	99.3
3	2.6	0.1	7.2/0.03	99.6
4	4.6	15.0	7.2/0.04	99.4
5	Not demonstrated	0	7.2/0.06	99.2
6	5.6	100.0	7.2/0.02	99.8
7	5.5	100.0	7.2/0.03	99.6
8	4.3	8.0	7.2/0.07	99.1

* See Table 1.

** Virus titre in the culture fluid before purification was 5.7 log LD_{50}/ml in all samples. Virus titre after purification was determined in the fraction with maximal virus content.

***Virus yield was calculated by the formula given in *Materials and Methods.*

the fact that the pore diameter of the silicates was not greater than 0.36 μm; in the course of gel chromatography the virions did not penetrate into the pores (Mchedlishvili *et al.*, 1975).

Preparative gel chromatography

Based on the results obtained on analytical columns, for preparative gel chromatography of rabies virus we selected porous glass with a pore diameter of about 0.2 μm and a porosity of 1.6 cm^3/g, chemically modified by human albumin. Complete separation of virus from ballast proteins was thus secured. Results of several experiments are presented in Table 3.

The greatest volume of virus material that could be purified by gel chromatography on the column used (5.6 × 85 cm) was 400 ml. The concentration of ballast proteins in the purified preparation did not exceed 4 μg/ml as compared with 2—3 mg/ml in the starting crude suspension. Based on direct counts in the electron microscope, the amount of viral protein corresponded to up to 5% of the total protein content of the purified preparations.

Table 3. Gel filtration of rabies virus on preparative colums

Volume of virus sample (ml)	Virus titre before purification	($\log LD_{50}$/ml) after* purification	Protein contents** μg/ml I	Protein contents** μg/ml II	Virus yield %	Protein contents in starting material mg/ml
110	6.2	5.0	2	0.01	100	2.4
150	6.0	6.0	4	0.01	100	1.8
200	6.0	5.9	3	0.02	100	1.8
300	6.8	6.4	4	0.03	95	2.1
400	6.8	6.7	2	0.1	100	2.1
480	6.2	6.1	12	0.003	100	2.4

* See Table 2.

** Total protein (I) and virus protein (II) in the fraction with purified virus.

Fig. 1 illustrates purification by gel chromatography of infectious (I) and UV-inactivated (II) rabies virus. The yield of either infectious or inactivated virus was 100%. The maintenance volume (the elution volume of the given substance from the column) for either kind of virus was 900 ml (44% of the void volume of the column), while the maintenance volume for ballast proteins was 1800 ml. Elution of antigen only in the free column volume indicates that in our experiments complete virions were the carriers of immunogenicity. This assumption was confirmed by electron microscopy (Figs 2—5).

Concentration of rabies virus

We also attempted to use chemically modified MPG for adsorption chromatography of rabies virus. Oxypropyl-MPG, from which 0.1% of virus was eluted on gel chromatography (see Table 2), was selected as adsorbent.

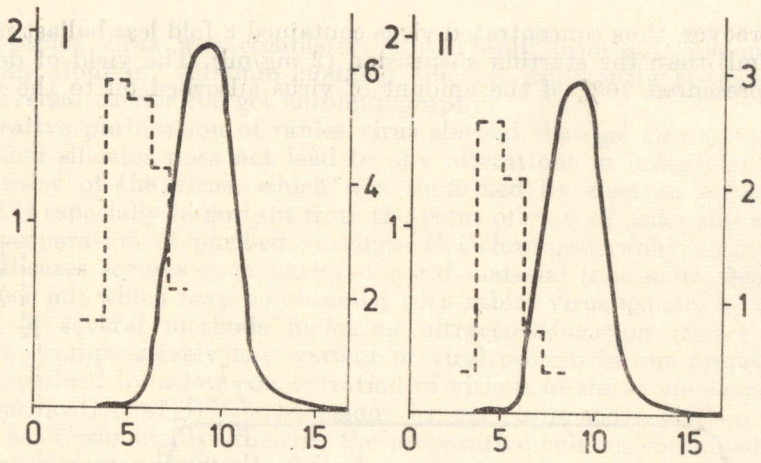


Fig. 1.

Gel chromatography of rabies virus on a preparative column of MPG chemically modified with human albumin

I — 300 ml of infectious virus suspension with a titre of 6 log LD_{50}/ml and E_{280} = 6.3 were placed on the column.

II — 300 ml of inactivated virus with an immunogenicity index of 2.7 and E_{280} = 4.2 were placed on the column.

Abscissae: fraction numbers; left ordinates: E_{280} values; right ordinates: virus titre in log LD_{50}/ml values (I) or immunogenicity index (II)

– – – – – Virus titre (I) or immunogenicity index (II)

——————— Optical density (E_{280})

One of the experiments is illustrated in Fig. 6. The procedure consisted of three stages. In the first stage, virus was adsorbed on to the carrier. To this end viral suspension was passed through the column for as long as the virions were adsorbed on to the surface of the carrier, i.e. until there remained free adsorption centres. In our case this corresponded to 0—80 ml of the eluate. After 80 ml had passed through the column, the titre of virus in the eluate became equal to that in the starting suspension, i.e. saturation of the adsorbent had occurred. The second stage of adsorption chromatography consisted of washing the virus-containing fluid out from the column. The column was washed with a buffer at sufficiently low pH to avoid desorption of the virus. In Fig. 6, this stage corresponded to 80—100 ml of the eluate (onset indicated by arrow). In the final stage, the adsorbed virus was eluted. This may be achieved by buffers at a higher pH (pH 8.6 buffer in our experiments). The virus was eluted in a small volume of desorbing buffer resulting in its concentration. In Fig. 6, the virus desorption stage corresponded to 100—108 ml of the eluate (onset of desorption indicated by arrow). In the experiment illustrated in Fig. 6, we obtained a 2-ml fraction the titre of which was 10-fold higher than the titre of the starting viral suspen-

sion. Moreover, thus concentrated virus contained 8-fold less ballast proteins (240 μg/ml) than the starting suspension (2 mg/ml). The yield of desorbed virus represented 70% of the amount of virus adsorbed on to the carrier.

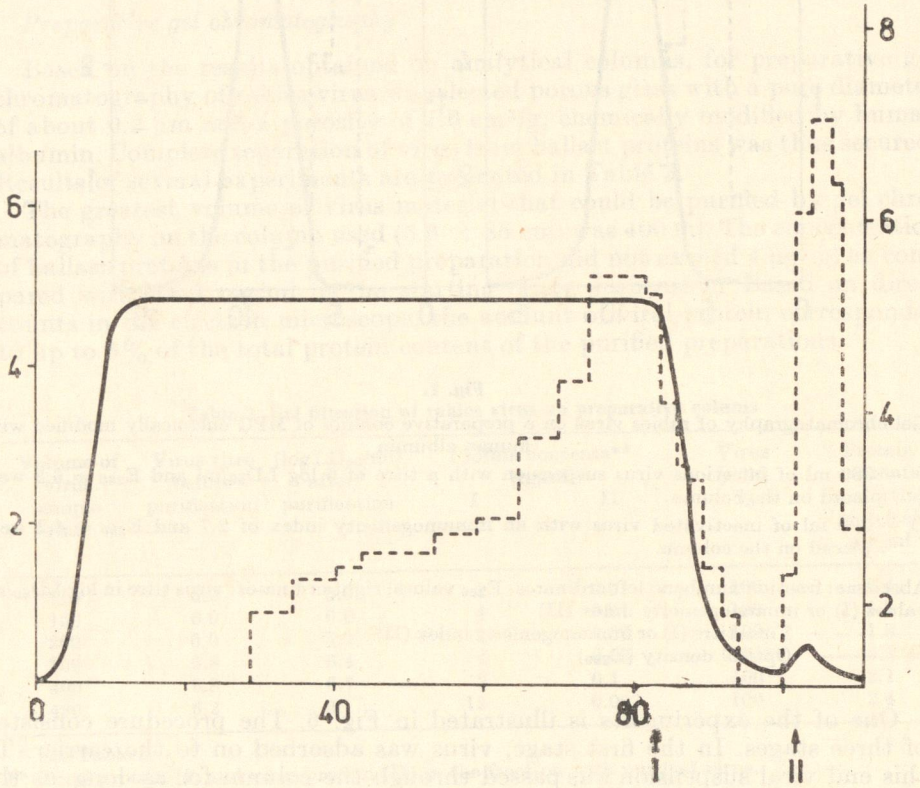


Fig. 6.

Adsorption chromatography of rabies virus

Eighty ml of a virus suspension with a titre of 5.8 log LD_{50}/ml and E_{280} = 5.1 were placed on a 0.8 × 5.0 column.

Abscissa: eluted volume (ml); left ordinate: E_{280} values; right ordinate: virus titre in log LD_{50}/ml values

– – – – Virus titre, ———— optical density (E_{280})

Arrows: start of virus elution by elution (I) and desorption (II) buffer

Discussion

Chemically modified MPG were used for purification of influenza (Mchedlishvili *et al.*, 1976), tick-borne encephalitis (Krasilnikov *et al.*, 1977) and Mengo (Gschwender and Traub, 1978) viruses. In all cases, the highest yields of purified virus were obtained with protein-modified glasses, in particular with those modified by human albumin. In our experiments, too,

purified rabies virus was completely eluted only from silicates modified by human albumin. Albumin-modified silicates apparently represent the most universal carrier for gel chromatography.

Preparative purification of rabies virus showed that gel chromatography on modified silicates does not lead to any alterations in infectivity or immunogenicity of the virus, which was confirmed by electron microscopy. This fact is especially important from the point of view of using this method for the preparation of purified vaccines. Gel chromatography on modified porous silicates secures such purity of viral material (not more than 4 μg protein per ml) which may be obtained with rabies virus usually by a combination of several methods including ultracentrifugation (Sokol *et al.*, 1968). The comparatively low content of viral protein in our preparations can be explained by a low concentration of virions in the crude suspension.

The purification of viral suspensions by gel chromatography on a preparative scale was highly efficient: the preparative column contained about 2 l of the carrier and 400 ml of a rabies virus suspension could be purified in one chromatographic cycle.

We also showed that chemically modified porous silicates can be used not only for gel chromatography, but also for adsorption chromatography of viruses. With the use of porous glasses with chemically bound OH groups of alcoholic character (oxypropyl MPG) for adsorption chromatography we reached a 10-fold concentration of rabies virus along with its partial purification from proteins. So far, rabies virus had not been concentrated on porous silicates due to its irreversible adsorption or inactivation (Hiatt *et al.*, 1971; Mchedlishvili *et al.*, 1972). In our experiments, rabies virus was eluted from oxypropyl MPG under mild conditions (0.1 M Tris-HCl, 0.2 M NaCl, pH 8.6). In this way, as shown by infectivity titration and electron microscopy, the virus particles were well preserved.

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Explanation of Electron Micrographs (Plate XXXVI):

Fig. 2. — Starting suspension of rabies virus (culture fluid). × 50 000.
Fig. 3. — Inactivated preparation of rabies virus purified by gel chromatography. × 50 000.
Fig. 4. — Rabies virus preparation concentrated by adsorption chromatography. × 80 000.
Fig. 5. — A rabies virion from the concentrated preparation. × 200 000.

Negative staining of the preparations with 2% phosphotungstic acid, pH 7, was carried out on carbon-coated grids.