Journal of Chromatography, 130 (1977) 275–280

CHROM. 9357

ADSORPTION CHROMATOGRAPHY OF VIRUSES

S. E. BRESLER, N. V. KATUSHKINA, V. M. KOLIKOV, J. L. POTOKIN and G. N. VINOGRADSKAYA

Leningrad Institute of Nuclear Physics, Leningrad Polytechnic Institute, Leningrad (U.S.S.R.)

(First received February 13th, 1976; revised manuscript received May 18th, 1976)

SUMMARY

The adsorption isotherm of influenza viruses on wide-pore glass was measured and was found to conform to Langmuir's equation. The affinity constants were estimated at three temperatures and the free energy, enthalpy and entropy of adsorption were calculated. A detailed study showed that the transition from adsorption to total elution is very sharp and proceeds under critical conditions of pH near the pK of the SiOH groups. Therefore, conditions are very favourable for the elution chromatography of viruses. An efficient procedure for the purification and simultaneous concentration of influenza viruses was developed.

INTRODUCTION

The preparation of large stocks of purified and concentrated viruses is important for the production of antivirial vaccines. Methods generally used are technologically unsuitable (ultracentrifuge) or lead to dilution instead of concentration (gel filtration on Sepharose and agarose gels). The existence of appropriate adsorbents (wide-pore glass and silica gel) suitable for the development of chromatographic processes for the purification of viruses is demonstrated in this paper. In spite of the complicated structure of viruses and their large molecular mass (the influenza virus has a "molecular weight" of $250 \cdot 10^6$ daltons), they are reversibly adsorbed on silicates. Therefore, frontal chromatography yields a 1000-fold purification of viruses and simultaneously a 10–20-fold concentration. The development of this process depended on our knowledge of the adsorption thermodynamics of viruses, which were studied in detail using influenza virus.

EXPERIMENTAL

Adsorbents

A powdered wide-pore glass developed by Zdanov[1] was used. The average radius of the pores is 850 Å, the specific volume is 0.9 ml/g and the grain dimensions are 0.06–0.1 mm.

Viruses

Allantoic cultures of influenza virus (strain A_2–MRC 11; A_2 Singapore) were used. The virus was cultivated in the allantoic cavity of 10–11-day hen embryo. The allantoic culture is a transparent yellow liquid with a protein concentration of 5–7 mg/ml and a titre of viruses of $2 \cdot 10^9$–10^{10} virions/ml, *i.e.*, 100–500 haemagglutination units per millilitre[2]. For the measurement of adsorption isotherms, the virus suspension was purified by elution chromatography as described below.

Measurement of virus concentration

The standard method of measurement uses the specific reaction of haemagglutination, *i.e.*, the aggregation of erythrocytes, when a definite volume of the virus suspension is added to a standard suspension of hen erythrocytes. This method is rapid and permits the measurement of the virus titre in about 1 h. The titre is expressed in haemagglutination units[2].

The absolute concentration of virus particles was measured by direct counting in an electron microscope[3]. Polystyrene latex and bacteriophage T1 served as internal standards; both gave identical results. It was shown that 1000 hemagglutination units per millilitre is equivalent to $2 \cdot 10^{10}$ virions/ml. The standard error in these measurements was $\pm$ 20%, due to statistical fluctuations.

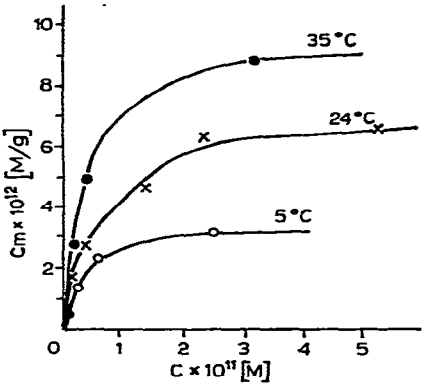
The stock of viruses was a standard one inoculated in embrionated eggs from one virus strain. The viability of viruses was controlled many times using a standard assay of mice infection. The purity of the final viral suspension was characterized by the ratio of the protein content (measured by Lowry's method) to the absolute titre of the virus. This ratio is well known for the pure influenza virus. The purity of our stocks varied in the range 40–70% (in terms of protein content).

Measurement of adsorption isotherms

We measured the equilibrium adsorption of viruses on wide-pore glass at 5°, 24° and 35° under static conditions. The time of equilibration was measured in advance and found to be about 1 h. Weighed samples of glass (25–50 mg) were introduced into virus suspensions of different concentration diluted with Tris–hydrochloric acid buffer (0.05 *M*, pH 7.4) and 0.15 *M* sodium chloride. The probe was stirred in a thermostat and after equilibrium had been attained the titre was measured. The difference between the initial and final titres gave the amount of virus adsorbed on the glass. The adsorption on glass walls was negligible (less than 5% of the total).

Elution of viruses from chromatographic columns

The influence of the eluent composition on virus elution was studied under dynamic conditions using a column of dimensions 20 cm $\times$ 1 cm^2. The column was filled with glass powder under a flow of buffer of the composition given above. A virus suspension (1 ml) was introduced into the column and eluted at a flow-rate of 1 ml/cm^2·min. At the outlet, a series of probes were sampled and titrated for virus content. The elution profile was plotted using these data and the total amount of eluted virus was evaluated.



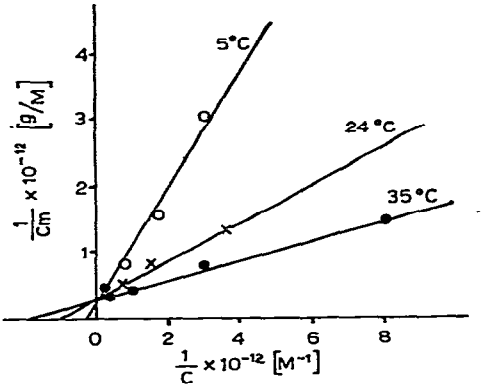


Fig. 1. Adsorption isotherms of viruses A_2–MRC 11 on wide-pore glass at different temperatures. The ordinate is the amount of virus in moles per gram of adsorbent.

Fig. 2. Adsorption isotherms plotted as inverse values $1/C_m$ and $1/c$ (according to Langmuir's equation); C_m and c are measured in moles of virus per gram of adsorbent or litre of solution, respectively.

RESULTS AND DISCUSSION

Thermodynamic characteristics of adsorption of viruses

In each series of experiments, adsorption isotherms at 5°, 24° and 35° were obtained using the same stock of influenza viruses. The solution contained Tris–hydrochloric acid buffer (pH 7.2–7.4). Under these conditions, the adsorption was reversible and the equilibrium value did not depend on the direction of approach.

Typical isotherms are presented in Fig. 1, and they all show saturation with increasing concentration. With increasing temperature, the amount of viruses adsorbed increased both on the linear part of the isotherm and on the plateau. The capacity of the wide-pore glass for viruses varies for different samples of glass in the range 10^{12}–$4 \cdot 10^{12}$ virions/g. All of the adsorption isotherms conform to Langmuir's equation, $C_m = ac/(k+c)$, where C_m is the equilibrium adsorption per gram, a the amount adsorbed at saturation, c the equilibrium concentration in solution and k the dissociation constant, *i.e.*, the inverse of the affinity constant. When plotted as inverse values, $1/C_m$ *versus* $1/C$, the isotherms are straight lines (Fig. 2). The value of the dissociation constant, k, is the concentration of the solute at half saturation. For different stocks of purified viruses, k varies in the range $1.4 \cdot 10^9$–$2.4 \cdot 10^9$ virons/ml, *i.e.*, between $2.3 \cdot 10^{-12}$ and $4 \cdot 10^{-12}$ M. We shall apply the notation of molar concentration to viruses, defining a molar solution in the usual way as that which contains $6 \cdot 10^{23}$ particles (Avogadro's number) per litre. The variations of the dissociation constant, k, for different stocks are understandable. Of course, for such a material as viruses, it is the order of magnitude that matters. The extremely low values of the dissociation constant show the enormous affinity of the sorbent towards the viruses. The limits for the value of k depend on the variable degree of purification of the viruses.

As is well known,

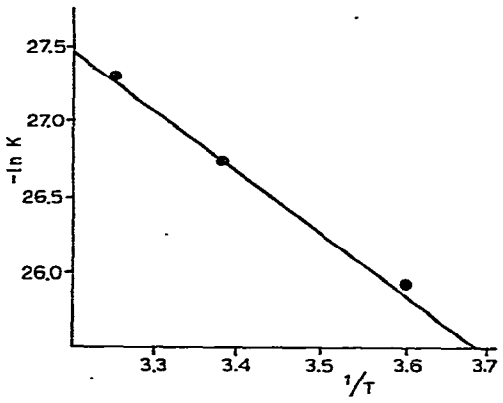
$$\ln k = \frac{\Delta F}{RT} = -\frac{\Delta S}{R} + \frac{\Delta H}{R} \cdot \frac{1}{T} \qquad (1)$$

where ΔF is the variation of free energy during adsorption (cal/mole), ΔS the variation of entropy, ΔH the variation of enthalpy and R the gas constant.

Fig. 3 shows a plot of ln k *versus* $1/T$ for the three temperatures studied (all measurements for one stock of viruses), and Table I gives the thermodynamic data calculated from this graph.

All experiments gave a decrease of 16 000–18 000 cal/mole for the free energy of the sorbate, while the enthalpy of viruses increased during adsorption by 6000–8000 cal/mole. This situation is typical of the binding of non-polar particles in aqueous media. As was originally shown by Nemethy and Scheraga[4], the main contribution to the free energy of interaction is due to an increase in the entropy of water because the disappearance of the interfaces between hydrophobic particles and water results in disorientation and disordering of the water molecules. The resulting interactions are called hydrophobic forces, although their source is not an energy but an entropy variation.

The systematic increase in the height of the plateau with temperature is readily explained by the competition of viruses with different impurities for the surface of glass. The total amount of impurities adsorbed is about 50%. Therefore, the competition for the surface is strong and elevated temperatures are advantageous for the adsorption of viruses because of the predominance of hydrophobic forces. The affinity constant of viruses on glass (*ca.* 10^{12} l/mole) is 10^7 times that of ordinary low-molecular-weight sorbates (10^2–10^5 l/mole), because a virion is attached to the surface by means of tens of bonds formed by the subunits of the virus capsid.



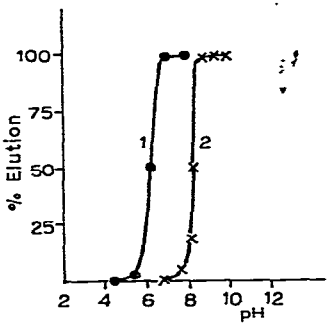


Fig. 3. Temperature dependence of the affinity constant of virus to glass. 1/T values are $\times 10^{-3}$.

Fig. 4. pH dependence of the desorption of influenza viruses from wide-pore glass. ●, strain $A_0/32/49$; ×, strain $A_2/2226/61$.

TABLE I

THERMODYNAMIC DATA CALCULATED FROM FIGS. 2 AND 3

The affinity constant, k, was determined from the linear plot in Fig. 2, using the slope of the straight lines (equal to K/a) and the initial ordinate, $1/a$. The values of ΔS, ΔH and ΔF were calculated from eqn. 1, using the plots in Fig. 3.

t(°C)	$1/k$	ΔS (cal/mole·deg)	ΔH (cal/mole)	ΔF (cal/mole)
5	$2.0 \cdot 10^{11}$	78.5	7300	−17300
24	$4.5 \cdot 10^{11}$			
35	$7.5 \cdot 10^{11}$			

Elution of viruses from chromatographic columns

Viruses are adsorbed on wide-pore glass at acidic or neutral pH, *i.e.*, under conditions when the glass surface is not charged. The isoelectric points of most viruses are in the pH range 4–5. The virions are negatively charged at neutral pH but their adsorption is due mainly to hydrophobic forces and, to some extent, to hydrogen bonds. At alkaline pH, the glass surface becomes negatively charged (the pK value of SiOH groups is 8.2). Therefore, electrostatic repulsion arises between viruses and the glass surface and its energy increases with increasing pH because of the gradual increase of the number of ionized groups on the glass. Finally, a critical point is attained when the repulsion forces are just equal to the cohesion and under these conditions there is a very abrupt transition from total adsorption to total elution (Fig. 4). We propose to call this process "critical adsorption" to emphasize its difference from ordinary adsorption of low-molecular weight substances. The transition from adsorption to elution occurs in a pH interval of 0.1–0.2 unit, which gives an "all or none" appearance to the phenomenon. This peculiarity is clearly due to the high free energy of the adsorption process (18,000 cal/mole). The spreading of elution conditions depends on thermal fluctuations, which equal $kT/2$ or 300 cal/mole at ordinary temperatures. This value is only 2% of the free energy of adsorption and therefore the sharpness of desorption is natural. This property is very favourable for the elution chromatography of viruses because it prevents tailing of chromatographic zones and makes the elution extremely sharp.

For another strain of influenza virus the transition point is shifted to the acidic side by two units. In some instances the coulombic repulsion is not sufficient for the desorption of viruses from glass, especially when hydrogen bonds between glass and the virus capsid are important (polio viruses). In this instance the addition of urea to the elution medium is efficient[5].

Interesting variations were found in the action of ionic strength or neutral electrolytes. Near neutral conditions (pH 6–8), moderate concentrations of sodium chloride (0.1–0.2 M) are favourable for adsorption, probably because of screening of electrostatic charges. At higher concentrations of sodium chloride (0.5–1 M), it acts in the opposite direction, effecting elution (Fig. 5). Probably Si–OH groups on the surface are converted into Si–ONa as a result of ionic exchange. The latter are unable to form hydrogen bonds with the protein of the virus capsid and therefore elution is effected at pH 7.3 instead of pH 8.

Purification of viruses by elution chromatography

The study of the adsorption and elution of viruses made it possible to use this process for the purification and concentration of viruses. An example is presented in Fig. 6, which shows the elution profile of influenza virus A_2 Singapore. The virus suspension was an allantoic culture of influenza viruses and the initial titre was about $5 \cdot 10^9$ virions/ml. The glass column had the dimensions 20 × 2.6 cm and volume of 750 ml of the allantoic suspension was introduced into it. Virtually the whole surface was saturated with viruses because a breakthrough of virus appeared at the outlet. The column was then washed with a solution of pH 7 and elution of viruses was effected by means of Tris–hydrochloric acid buffer of pH 8.5 at a rate 1 ml/cm^2·min. As shown in Fig. 6, the virus is eluted in a narrow peak and its concentration is 20 times higher than that in the initial suspension. The degree of purity increases 800-

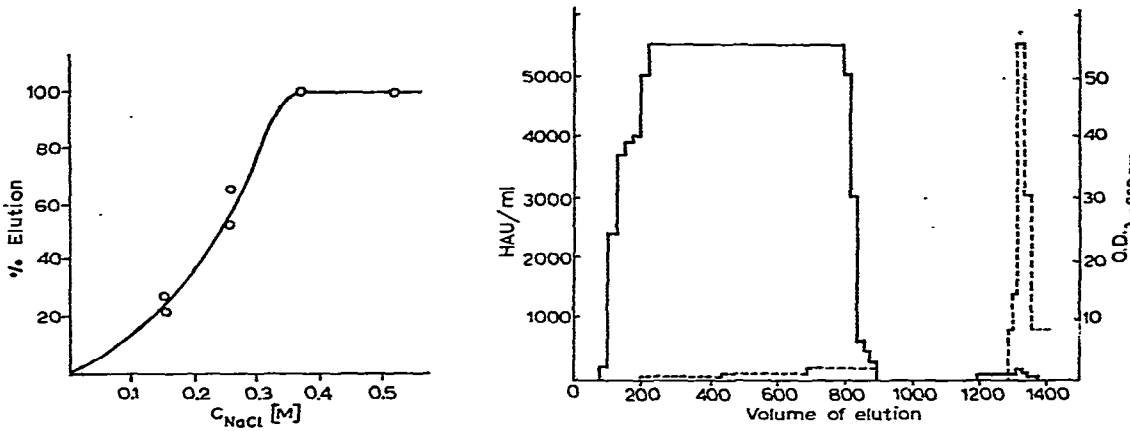


Fig. 5. Influence of the concentration of NaCl in the eluting buffer (0.05 *M* Tris, pH 7.3) on the desorption of influenza virus B (U.S.S.R.) 69 from wide-pore glass.

Fig. 6. Chromatographic profiles during the adsorption (left) and elution (right) of influenza virus A_2 Singapore from a column of wide-pore glass. ———, Concentration of proteins during elution at the outlet of the column measured by $O.D._{280\,nm}$; ---------, titre of the virus during elution (haemagglutination units/ml).

fold and attains about 40% of the theoretical limit. The total amount of viruses eluted is equal, within the limits of experimental error, to the amount introduced with the allantoic fluid. Hence the separation of viruses from impurities on porous glass is very efficient and the biological activity is entirely preserved.

CONCLUSIONS

The direct measurement of the adsorption isotherm of influenza viruses on wide-pore glass gave a value of *ca.* 10^{12} l/mole for the affinity constant and 18,000 cal/mole for the free energy of adsorption. The interaction is due mainly to hydrophobic forces and yields a strong increase in entropy.

Elution of adsorbed viruses from chromatographic columns is effected sharply if the pH of the medium is increased to about 8 (depending on the specific virus used). Desorption is due to electrostatic repulsion of anionic charges on the virions and glass. The critical conditions of elution are very favourable for elution chromatography because very sharp zones are formed and tailing is prevented.

Applying column chromatography to allantoic cultures of viruses, it is possible to purify them 1000-fold in one run and to concentrate them simultaneously about 20-fold. For influenza viruses, a suspension of 50% purity was prepared according to this procedure. Even different strains of the same virus can be separated using this method.

ACKNOWLEDGEMENT

We thank Dr. Mosevitsky for help and advice.

REFERENCES

1 S. P. Zdanov, *Dokl. Akad. Nauk SSSR*, 28 (1952) 281.
2 J. K. Hirst, *J. Exp. Med.*, (1941) 75.
3 R. C. Backus and R. C. Williams, *J. Appl. Phys.*, 21 (1950) 11.
4 G. Nemethy and H. Scheraga, *J. Phys. Chem.*, 66 (1962) 1773.
5 S. Bresler, V. Kolikov, N. Katushkina, M. Chumakov and S. Zhdanov, *Virology*, 59 (1974) 36.