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PURIFICATION OF INFECTIOUS RUBELLA VIRUS BY GEL FILTRATION ON SEPHAROSE 2B COMPARED TO GRADIENT CENTRIFUGATION IN SUCROSE, SODIUM METRIZOATE AND METRIZAMIDE

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Rubella virus was purified by chromatography on Sepharose 2B after concentration by ultrafiltration on hollow fibers and hydroextraction with PEG 20,000. Yields of 40% infectivity and 70% hemagglutinating activity were routinely obtained. Chromatographic purification was compared to ultracentrifugation in sucrose, metrizamide and sodium metrizoate. Yields were lower in sucrose and metrizamide, while sodium metrizoate reduced the infectivity of the virus below detectable levels. These results demonstrate the advantage of Sepharose 2B for the purification of infectious rubella virus.

INTRODUCTION

Density gradient centrifugation in sucrose is applied to the purification of viruses with varying degrees of success (Hinton and Dobrota, 1976). Iodinated aromatic compounds such as sodium metrizoate or metrizamide have also been suggested for density gradient purification of labile viruses (Hinton and Mullock, 1976). These compounds are primarily used as X-ray contrast media. Sodium metrizoate is a derivative of triiodo-benzoic acid; this medium has been used for the separation of cells and microorganisms but is unstable in certain conditions (pH, ionic strength) (Hinton and Dobrota, 1976; Hinton and Mullock, 1976). A recently introduced X-ray contrast medium, metrizamide (2-[3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido]-2-deoxy-D-glucose) is a completely covalent molecule and, as such, forms stable density solutions up to 1.45 g/ml. These solutions are more mobile than equivalent solutions of sucrose or Ficoll. Metrizamide and related compounds should prove useful in situations where more conventional media give poor results (Hinton and Dobrota, 1976).

The method of choice for the purification of rubella virus has always been sucrose density gradient (Vaheri et al., 1969; Liebhaber and Gross, 1972; Payment et al., 1975; Trudel and Payment, 1980). Infectious titer yields for purification by one cycle isopycnic banding has been in the range of 15%. Further purification on a second gradient reduces the yield to less than 1% (Vaheri et al., 1969). In earlier studies, salt gradients (potassium

tartrate or citrate, cesium chloride or sulfate) were tried, but also showed problems with regard to recovery of infectivity.

On the other hand, exclusion chromatography of rubella virus on Sephadex G-200 has been reported (Veronelli and Maassab, 1965; Schmidt and Lennette, 1966) and Sepharose 2B gel filtration has been used successfully for the purification of other viruses (Bengtsson and Philipson, 1964). More recently, Sepharose gel filtration has been applied to the purification of hepatitis A (Hornbeck et al., 1975/76; Locarnini et al., 1978), poliovirus (Van Wezel et al., 1978) and coronaviruses (Ignatov et al., 1979) with efficiencies up to 85%. This report describes the application of Sepharose 2B gel fitration for the purification of infectious rubella virus in comparison to centrifugation on sucrose, metrizamide and sodium metrizoate.

MATERIAL AND METHODS

Virus production

Rubella virus, strain M-33 (ATCC VR-315) was produced on monolayers of Vero cells grown in Corbeil Bellco TM system (Bellco Glass Co., Vineland, NJ) (Corbeil et al., 1979). Cells were cultivated in one TM5 module in equal parts of Medium 199 (Hanks' base) and minimal essential medium (Earle's base) supplemented with 4% kaolin-treated calf serum (treated to remove non-specific inhibitors of hemagglutination) and 50 μg/ml of gentamicin.

Virus concentration

Two liters of harvested supernatant containing 16 HAU/0.025 ml (hemagglutinating units) were concentrated by hollow fiber ultrafiltration on an Amicon DH-4 model system (Amicon, MA). The ultrafiltration system was modified with quick-connect fittings and air filters to minimize aerosol dispersion. The concentrator was equipped with an H1-100 hollow fiber cartridge (molecular weight cut-off of 100,000) and operated according to manufacturer specifications. Minimal volume after DH-4 concentration is 75 ml and concentration efficiency between 75% and 90% (Trudel and Payment, 1980). The concentrate was separated in aliquots of 5 ml and stored at -70°C.

Virus purification

Ultracentrifugation. Concentrated rubella virus was disaggregated by adding 10 mM EDTA and the suspension was clarified by centrifugation at 15,000 *g* for 20 min at 4°C. Aliquots of 5 ml of viruses were purified by two cycles of density gradient centrifugation in sucrose, metrizoic acid (sodium salt) or metrizamide (Sigma Chemical Co., St. Louis, MO). The virus was layered on a discontinuous 30%/50% (5 ml/5 ml) gradient and centrifuged at 35,000 r.p.m. in a SW-40 rotor for 3 h at 4°C. The virus-containing

fractions were diluted 1 : 3 with NTE buffer (0.15 M NaCl; 0.05 M Tris HCl; 0.001 M EDTA; pH 7.4), relayered on a preformed 15–50% linear gradient and centrifuged to equilibrium (SW-40, 35,000 r.p.m., 18 h). Gradients were analyzed with an Isco model 640 fractionator and a model UA-5 absorbance monitor. All gradients were prepared in NTE buffer.

Chromatography. Five ml of concentrated rubella virus were made 10 mM EDTA, clarified by centrifugation at 10,000 *g* for 15 min and concentrated to 1 ml by hydroextraction in dialysis tubing with PEG 20,000 (Carbowax 20M, Union Carbide, Montréal, Canada). This material was purified by gel filtration on Sepharose 2B (Pharmacia Fine Chemicals, Montréal, Canada) in a 2.5 X 40 cm column. NTE buffer was used as the eluent at a flow rate of 10 ml/h. Fractions (3 ml) were collected and assayed for infectivity, hemagglutinating activity and optical density at 280 nm.

Virus assay

Hemagglutinin titer. Assays for hemagglutinating activity were performed in Cooke Engineering microtiter round bottom disposable plastic plates. Serial twofold dilutions of virus were made in 0.025 ml volumes of modified Auletta buffer (0.9% NaCl, 0.1% $CaCl_2$, 0.1% $MgSO_4 \cdot 7H_2O$, 1% kaolin-treated fetal bovine serum). Hemagglutinating activity was revealed by adding 0.050 ml of a 0.25% one-day-old chick erythrocyte suspension (Trudel et al., 1979). The plates were incubated at 4°C for 1 h. One unit of antigen was defined as the highest dilution that produced a complete hemagglutination.

Infectivity titer. Virus preparations were titrated by interference with Echo 11 virus on primary cercopithecus monkey kidney cells (Furesz et al., 1969).

Protein. Protein concentration was determined using the Bio-Rad protein assay procedure (Bio-Rad Laboratories, CA).

Electron microscopy. Fractions, positive by hemagglutination, were examined after negative staining with phosphotungstic acid 3%, pH 6, with a Phillips EM 300 microscope.

Osmolality. Osmolality was measured with an Osmette (Precision Systems, Inc., MA).

RESULTS

Infectious tissue culture fluids were concentrated by hollow fiber ultrafiltration with an efficiency of 76.8% as previously reported (Trudel and Payment, 1980). Aliquots were

TABLE 1

Stability of rubella virus infectivity at 4°C in different purification media[a]

Time (h)	NTE buffer	Sucrose	Metrizoate	Metrizamide
0	8.9	8.9	8.9	8.9
2	8.6	8.75	7.6	8.70
24	8.45	8.8	0.5	8.4

[a] Titers are expressed in $\log_{10}$ and are the mean of three experiments.

analyzed by ultracentrifugation in sucrose, sodium metrizoate and metrizamide, and chromatography on Sepharose 2B with NTE buffer.

We first studied the stability of rubella virus infectivity in these media. As can be seen from Table 1, after 2 h there is an average inactivation of 40–50% in NTE buffer, sucrose and metrizamide. In sodium metrizoate inactivation reached 95%. After 24 h, virus inactivation was higher in NTE buffer and metrizamide than in sucrose. In sodium metrizoate inactivation was nearly complete.

We also measured the osmolality of the solutions used for separation, reasoning that physiological osmolality should prove more suitable for viral purification. The osmolality of NTE buffer and tissue culture medium is within physiological range (260–320 mOsm) while metrizamide is slightly higher (458 mOsm). Both sucrose and sodium metrizoate are over 2000 mOsm which was the limit of our measuring osmometer.

Density gradient centrifugation of rubella virus on sucrose, metrizamide or sodium

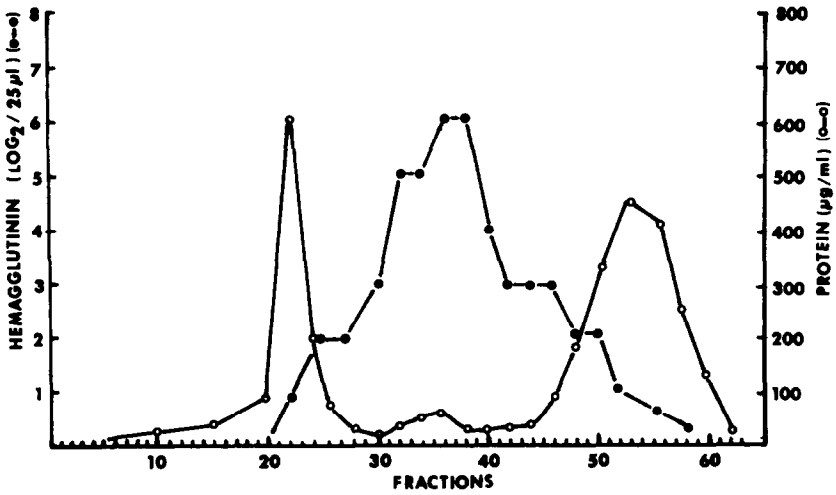

Fig. 1. Chromatography of rubella virus of Sepharose 2B 2.5 × 40 cm column. Eluent: NTE buffer pH 7.4. Flow rate: 10 ml/h. Fraction size: 3 ml.

TABLE 2

Purification by ultracentrifugation and chromatography of concentrated rubella virus[a]

	Molecular weight of separation media	Osmolality[b] (mOsm)	Buoyant density of virus (g/cm^3)	Recovery	
				HAU	$TCID_{50}$
Concentrated virus (5 ml)	NA	NA	NA	65,566 (100%)	$10^{9.6}$ (100%)
Ultracentrifugation[c]					
Sucrose	342.3	> 2000	1.19	40,960 (62%)	$10^{8.83}$ (16.8%)
Metrizamide	789	458	1.19	40.960 (62%)	$10^{8.71}$ (13%)
Sodium metrizoate	628	> 2000	1.19	10,240 (15%)	$10^{1.6}$ (0.01%)
Chromatography[d]					
Sepharose 2B	NA	NA	–	46,080 (70%)	$10^{9.2}$ (40%)

[a] Virus (5 ml, 65,566 HAU and $10^{9.6}$ $TCID_{50}$) was purified by ultracentrifugation or chromatography as described in Material and methods.

[b] Sucrose, metrizamide and sodium metrizoate osmolality was measured on 40% (w/w) solutions.

[c] All solutions were prepared in NTE buffer (280 mOsm).

[d] NTE buffer was used as eluate for chromatography purification of rubella virus.

NA: not applicable.

metrizoate yielded similar isopycnic patterns with similar buoyant densities at 1.19 g/cm^3 (Table 2). Recovery of hemagglutinating activity was equivalent for sucrose and metrizamide (62%) and much lower for sodium metrizoate. Infectivity yields paralleled these findings, except that infectivity was below detectable level with sodium metrizoate.

Chromatography on Sepharose 2B gave better recovery of hemagglutinating activity (70%) and infectivity (40%) (Table 2, Fig. 1) than ultracentrifugation. Purified virus was recovered in eight fractions (fractions 31–38) that corresponded to one of the protein peaks (Fig. 1). Three protein peaks are resolved (fractions 1) 20–26, 2) 31–38 and 3) 46–62). Peak 1 consisted of cellular debris of over 40×10^6 molecular weight that

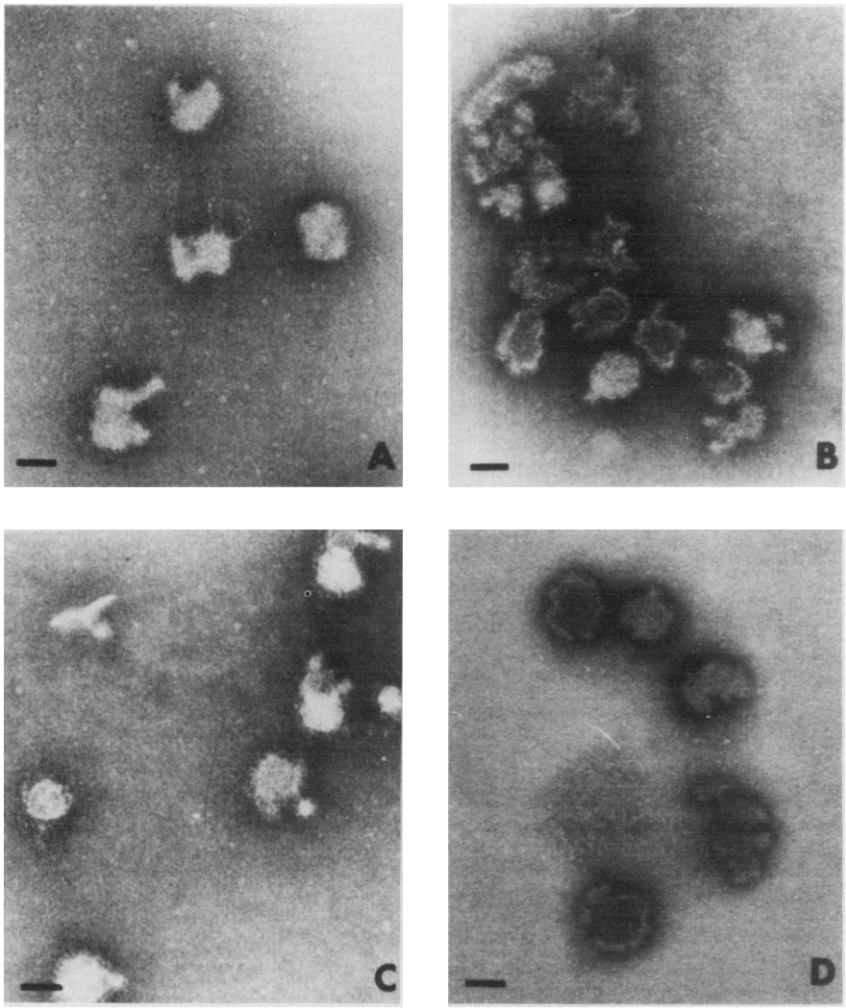

Fig. 2. Electron micrograph of rubella virus purified by isopycnic banding in sucrose (a), sodium metrizoate (b), metrizamide (c) and chromatography on Sepharose 2B (d). Scale bar is 50 nm.

migrated with void volume. Peak 2 was the virus. Peak 3 consisted of low molecular weight proteins.

Electron microscopic examination of viruses, purified by the four methods, revealed good structural preservation by chromatography (Fig. 2D), sucrose (Fig. 2A), and metrizamide (Fig. 2C), while sodium metrizoate purified viruses (Fig. 2B) revealed penetrated virions and less defined hemagglutinins at the membrane surface. Viruses purified in metrizamide and sucrose were about one-third smaller than those purified by chromatography and had a more relaxed viral configuration especially visible on viruses purified in metrizamide (Fig. 2B). Viruses purified by chromatography on Sepharose 2B (Fig. 2B) showed a more compact structure and a denser hemagglutinin border. Pleomorphic forms were also more evident than by centrifugation.

DISCUSSION

Purification of rubella virus by multi-step ultracentrifugation has been difficult to achieve because of the fragility of rubella virus. Yields of infectious virions of 0.1–15% have been reported (Vaheri et al., 1969). Our study reports similar yields (16%). These results indicate the fragility of rubella virus to repeated sucrose gradient centrifugation. On the other hand, iodinated density gradient media have been recommended for the purification of labile viruses because of their low osmolality (Hinton and Mullock, 1976). Purification of rubella virus yielded similar results in sucrose and metrizamide but the infectivity was reduced to undetectable levels in sodium metrizoate. For sodium metrizoate, this was expected as it inactivates the virus infectivity (Table 1), although hemaglutinating activity is not affected. Similar results have been reported for herpes simplex virus, where 10–30% of infectivity was recovered by metrizamide gradient centrifugation, while infectivity was not detected after sodium metrizoate purification (Blomberg et al., 1976).

Exclusion chromatography of rubella virus on Sephadex G-200 has been reported to permit recovery of total infectivity (Veronelli and Maassab, 1965; Schmidt and Lennette, 1966) but did not permit separation of virus from protein and particles of 600,000 molecular weight. Improved separation has been mentioned by Vaheri reportedly using Sepharose 4B, but no results were published. Sepharose 4B (Pharmacia Fine Chemicals) has a fractionation range of 6×10^4–20×10^6 which does not permit separation because the virus migrates with the void volume. Sepharose 2B, with its exclusion limit of 40×10^6 molecular weight permits fractionation of the virus which is slowed by the Sepharose beads and is separated from the void volume contaminants.

Pearl agar chromatography has been successfully applied to the purification of viruses with recoveries of 50–82% (Bengtsson and Philipson, 1964). Sepharose 2B has been used to purify hepatitis A virus with yields of 80% (Locarnini et al., 1978). In our hands, chromatography of rubella virus on Sepharose 2B yields recovery of 70% of the virus-associated hemagglutinin and 40% of the infectivity in peak material. This is by far better than what has been reported so far for the purification of rubella virus. Furthermore,

total hemagglutinin recovery is 95–100% when taking in account fractions 42–50 where free hemagglutinin is eluted (Fig. 1). Chromatographic purification of rubella virus is routinely used in the laboratory and variations between experiments are within a normal range.

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