New Method for the Removal of Extraneous Proteins from Purified Oncornaviruses

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Conventional methods (i.e., gradient centrifugation) for the purification of oncornaviruses are usually not effective in complete removal of nonviral proteins. Such contaminants often prove to be a nuisance in subsequent immunological or biochemical studies. Hyperimmune sera prepared from these viruses must be absorbed to assure specificity; cell-derived proteins can be shown to interfere with studies of virus structural proteins, nucleic acids, or viral enzymes. Herein is described a method for removal of most of these contaminants. Viruses are diluted in a high concentration of NaCl to achieve a final concentration of 15%, incubated for 30 min, sedimented, and resuspended in buffer. This procedure results in reductions of up to 48% of the protein without affecting particle count. Immunological, biochemical, and biological properties are not adversely affected. Of the proteins removed, fetal calf serum components and a ribonuclease (presumably cell-derived) were identified. This technique differs significantly from other high-salt methods in that the virus is not precipitated from suspension. It is believed that absorbed proteins are desorbed and left in solution (or suspension) as the virus is sedimented by centrifugation.

The preparation of highly purified oncornaviruses has become an essential part of the molecular characterization of these agents. A principal problem in accomplishing this task has been the removal of nonviral proteins. This is especially true with tissue culture-grown viruses which often contain proteins derived from either the host cells or components of the culture medium, i.e., fetal calf serum (FCS). Most of the cell debris and culture medium is frequently removed by single or multiple bandings of the virus preparation in isopycnic density gradients (i.e., sucrose or cesium chloride). It is widely known, for example, that, even when gradient-purified virus preparations are employed as antigens to prepare hyperimmune sera, the resulting antiserum must be adsorbed with FCS, noninfected cells, etc., to render it virus specific.

The present communication describes a method for removing extraneous proteins from gradient-purified viruses by treatment with a high concentration of sodium chloride. This method differs from other methods using high salt concentrations, polyethylene glycol, or alcohol in that the virus is not precipitated.

MATERIALS AND METHODS

Viruses. Procedures for the preparation of Rauscher leukemia virus (RLV) grown in the JLS-V9 cell line (1, 2) and of RD-114 virus (RD) (4) have

been previously described. The gibbon ape lymphoma (GALV) and simian sarcoma viruses (SSV-1) were grown in chronically infected NC-37 cells (D. Larson, unpublished data) and purified from tissue culture fluids harvested 5 to 7 days after culture initiation. Tissue culture fluids harvested from all cultures were processed through a K-type continuous flow zonal ultracentrifuge using a linear (15 to 55%) isopycnic sucrose gradient. Virus bands were either pelleted at $70,000 \times g$ for 90 min (singly banded; RLV, SSV-1), or rebanded on a second, similar sucrose gradient prior to pelleting (doubly banded; GALV, RD). Virus preparations were resuspended in buffer consisting of 10 mM tris(hydroxymethyl)aminomethane (Tris), 100 mM sodium chloride, and 1 mM ethylenediaminetetraacetic acid, pH 7.4 (TNE), and stored at -70°C .

Salt treatment. Virus preparations were diluted with an equal volume of 5.3 M NaCl in TNE (pH 7.4) to achieve a final concentration of 2.6 M NaCl (15%). After incubation at 37°C (or 4°C) for 30 min, the virus was sedimented at $70,000 \times g$ for 90 min and resuspended in TNE. Parallel portions of each virus preparation were treated with TNE to serve as controls.

Electron microscopy. Electron microscopic evaluations of the treated and control preparations were performed by the negative staining method of Monroe and Brandt (10).

Protein analysis. Quantitative protein analyses of these preparations were done by the method of Lowry et al. (8) using a crystalline bovine albumin (Armour) standard series and using a Technicon autoanalyzer. Virus proteins were also examined by electrophoretic analysis on 10% polyacrylamide gels

containing 0.1% sodium dodecyl sulfate (SDS; 14). Equal amounts (100 μ g) of protein from either salt-treated or control preparations were disrupted and electrophoresed. After staining of the electrophoresed samples, the molecular weights of the virus proteins were determined by reference to 1 μ g each of standard proteins electrophoresed on parallel gels. The standard proteins employed included phosphorylase A, bovine serum albumin, ovalbumin, α -chymotrypsinogen, and cytochrome *c*, which have molecular weights of 94,000, 68,000, 43,000, 25,000, and 12,400, respectively.

Infectivity. The RLV was assayed for infectivity by the D56 murine sarcoma virus rescue assay (2) after storage for 6 months following salt treatment. The SSV-1 was assayed by focus formation on normal rat kidney cells (1). Additionally, direct assessment of the infection of salt-treated RD and SSV-1 viruses was performed by adding 1 ml of virus to 3×10^5 F-265 or NC-37 normal lymphoblastoid cells. These cultures were examined 14 to 21 days later by electron microscopy for the presence of budding virus.

Serologic testing. The microimmunodiffusion and complement fixation methods used to compare test and control preparations have been described previously (14). All virus preparations were disrupted with ether prior to serologic testing. Antiserum to FCS was prepared in New Zealand white rabbits by 4 weekly subcutaneous and intradermal inoculations with undiluted FCS (GIBCO). Antisera to RD virus, from Hartly guinea pigs, and antisera to RLV, from New Zealand white rabbits, were prepared by weekly injections of ether-treated or NaCl-ether-treated virus (footpad with complete Freund adjuvant then subcutaneously for 5 weeks). Antiserum to RLV was also obtained from Osborne-Mendel rats bearing transplantable RLV-induced tumors (14).

Assay for endogenous reverse transcriptase activity. Disrupted virus (25 μ l) was added to an enzyme reaction mixture containing, in a final volume of 100 μ l: 50 mM Tris-hydrochloride (pH 7.8); 320 μ M each of deoxyadenosine 5'-triphosphate, deoxycytidine 5'-triphosphate, and deoxyguanosine 5'-triphosphate, 2.5 mM magnesium acetate, 8 mM dithiothreitol, 13 mM NaCl, and 40 μ M [3 H]thymidine 5'-triphosphate ([3 H]dTTP) (specific activity 700 dpm/pmol). After 60 min at 37 C, duplicate 25- μ l aliquots were withdrawn and applied to 2.1-cm disks of Whatman 541 filter paper. The disks were washed four times in 5% trichloroacetic acid and two times in ethanol (5), dried, and counted in Toluene/NCS/Omnifluor (6).

RNAse assay. Ribonuclease (RNAse A, virus) sources were added to a reaction mixture containing, in a volume of 300 μ l: 50 mM Tris-hydrochloride (pH 7.8), 1 mM ethylenediaminetetraacetic acid, 10 mM dithiothreitol, 150 μ g of yeast transfer ribonucleic acid RNA, and 5,000 to 6,000 counts/min of 3 H-labeled RNA extracted from normal human lymphoblastoid (NC-37) cells. After 15 min at 37 C, reactions were terminated by chilling to 0 to 4 C and by addition of 300 μ l of 10% trichloroacetic acid. Samples were centrifuged at $8,000 \times g$ for 15 min.

Supernatant (200 μ l) was counted in BBS/Toluene (10% BBS-3, 0.5% Omnifluor in Toluene).

Assay for RNA-dependent deoxyribonucleic acid (DNA) polymerase activity and viral high-molecular-weight RNA (12). Virus pellets were suspended in 200 μ l of Nonidet P-40 (0.2% [wt/wt]) in TE buffer (50 mM Tris-hydrochloride, pH 7.8, 1 mM ethylenediaminetetraacetic acid). After 10 min at 0 to 4 C, 100 μ l of disrupted virus was added to an enzyme reaction mixture containing, in a final volume of 150 μ l: 50 mM Tris-hydrochloride (pH 7.8); 320 μ M each of deoxyadenosine 5'-triphosphate, deoxycytidine 5'-triphosphate, and deoxyguanosine 5'-triphosphate; 2.5 mM magnesium acetate; 8 mM dithiothreitol; 13 mM NaCl; and 1.5 mM [3 H]dTTP (specific activity 110,000 dpm/pmol). After 30 min at 37 C the reaction was stopped by the addition of 350 μ l of SDS, NaCl, and Pronase to final concentrations of 1%, 0.2 M, and 500 μ g/ml, respectively. After 30 min at 37 C, reaction products were precipitated by addition of 0.1 volume of 0.05 M Na_2HPO_4 (pH 7.8) and 0.06 M MgCl_2 , and an equal volume of ethanol (4). After 30 min at -20 C, precipitates were collected by centrifugation at $3,000 \times g$ for 8 min, drained, and dissolved in 25 μ l of 0.1 M ethylenediaminetetraacetic acid (pH 7.8) and 75 μ l of TE buffer containing 5 mg cytochrome *c* per ml.

Reaction products were separated from unreacted [3 H]dTTP by chromatography on columns (0.7 by 15 cm) of Bio Gel P-6 which had been swollen in and equilibrated with TE buffer. The effluent containing the reaction products and cytochrome *c* (about 0.5 ml) was collected and precipitated by addition of 0.1 volume of 3 M LiCl and 2 volumes of ethanol. After storage at -20 C for 16 h, precipitates were collected by centrifugation ($15,000 \times g$, 30 min), dried, and dissolved in TE buffer containing 1% SDS.

Samples were applied to a gel (0.5 by 7 cm) containing 2.0% acrylamide and 0.5% agarose (6) and electrophoresed for 30 min at 150 V (7.5 mA/gel). Gels were fractionated and fractions analyzed for radioactivity as previously described (6).

RESULTS

Much of the data presented was derived from work done with RD virus, but supporting data with other viruses, which were available for confirmatory results, indicate that the effects would be the same or similar for all oncornaviruses.

Protein analyses of most virus samples (Table 1) showed a marked reduction in the protein content after treatment with NaCl when compared to controls. Statistical analyses of the six samples of RD virus compared in Table 1 indicated that salt-treated samples had an average of 1.02 ± 0.12 mg of protein/ml ($\pm$ standard error). In contrast, control samples contained 1.48 ± 0.21 mg of protein/ml. This represents an average reduction in protein levels after salt treatment of RD virus of 30.8%. Similar analyses of the virus particles obtained indicated

TABLE 1. *Effect of salt treatment on protein content and particle count^a*

Virus and lot no.	Protein (mg/ml)			Virus count (VP/ml ^b)	
	Treated	Control	(%)	Treated	Control
RD-33	0.81	0.94	14	2.1×10^{11}	4.2×10^{11}
RD-48	0.96	1.19	19	2.9×10^{11}	1.0×10^{11}
RD-51	0.99	1.22	19	4.2×10^{11}	4.2×10^{11}
RD-74	1.55	2.20	30	3.4×10^{10}	5.6×10^{10}
RD-79	1.08	2.07	48	4.2×10^{11}	3.2×10^{11}
RD-85	0.74	1.25	41	1.4×10^{10}	1.4×10^{10}
GALV-299	0.22	0.42	48	2.6×10^8	7.0×10^7
RLV-230	2.45	3.06	20	1.4×10^{11}	7.0×10^{11}
RLV-234	1.65	2.80	41	1.0×10^{10}	3.5×10^{10}

^a All viruses were harvested 6 to 7 days after culture initiation and prepared by gradient centrifugation as described in the text. Parallel portions were salt- or sham-treated prior to protein and electron microscopic analysis.

^b VP, Viral articles.

that each milliliter of salt-treated and control samples contained an average virus particle count of $(2.31 \pm 0.73) \times 10^{11}$ and $(2.22 \pm 0.76) \times 10^{11}$, respectively. The percentage of reduction varied for different virus preparations but was consistent in repeat trials on a single preparation. Reductions in protein have ranged from 5 to 48% and appeared to be affected by two factors: the extent of prior purification and the length of time in culture prior to virus harvest. Single banded RLV, SSV-1, or GALV often showed a greater average protein loss (~30%) than did the double banded RD or GALV (~20%). Viruses harvested 1 to 2 days after culture initiation consistently showed less protein loss than did those harvested at 6 to 7 days. Multiple treatments of a single preparation resulted in only small additional reductions in protein (<3%), however, these were accompanied by losses of virus of 0.5 to 1 log/ml. No apparent differences were found when treatment was carried out at 4 C instead of 37 C.

Electron microscopic examination of treated and control preparations showed that there was no significant change in virus numbers (Table 1). The numerical fluctuations were within the experimental error. Furthermore, the treatment did not visibly affect the morphology of any of the viruses (Fig. 1), except with RD virus where the virions showed more pleomorphism than is normally found (13).

The results of infectivity analyses, expressed as focus-forming units per milliliter, are presented in Table 2. There was no change in infectivity titers of Rauscher virus and in only one instance was there a drop in the activity of SSV-1. Electron microscopic examination of

cells infected with either treated or control RD or SSV-1 preparations showed the presence of budding virus particles. Thus it appears that neither the salt treatment nor storage after treatment was detrimental to virus infectivity.

Figure 2 presents results of microimmunodiffusion tests performed with RD virus. Similar data, although not shown were obtained with RLV. Antiserum prepared against virus which had been only ether disrupted had a strong reactivity with FCS as compared to the reactivity with the viral antigens (Fig. 2a). Comparison of the reactivity of treated and control virus with anti-FCS (Fig. 2b) showed that FCS proteins present on untreated virus were removed by the treatment and could subsequently be identified in the dialyzed, concentrated NaCl supernatant. Antiserum prepared against salt-treated, ether-disrupted RD (Fig. 2c) showed a strong reactivity for the viral antigens and virtually no reactivity with FCS proteins (when compared with the serum in Fig. 1a).

The densities of treated and nontreated RLV, GALV, and RD were compared by linear isopycnic sucrose gradient centrifugation. The density of these viruses was found to increase slightly as a result of treatment. Shifts of 0.01 to 0.03 g/cm³ were observed after treatment: RD shifted from 1.15 to 1.17; RLV from 1.16 to 1.17; and GALV from 1.14 to 1.17 g/cm³.

The endogenous specific activity of the reverse transcriptase of all of these viruses was enhanced by salt treatment of the virus. Representative data for RD virus reverse transcriptase activity are shown in Table 3. This may be the result of either removal of inactive protein contaminant or the removal of adsorbed

FIG. 1. Morphologic appearance of sodium chloride-treated and control SSV-1 and RD virus. (A) SSV-1, treated; (B) RD, treated; (C) SSV-1, control; (D) RD, control. VP, Viral particles.

RNases which are able to degrade the RNA template for the enzyme reaction or both.

The removal of culture fluid derived proteins is described above. Table 4 shows that salt treatment caused a large loss in RNase activity of preparations of both RLV and GALV that was characteristic of all of the viruses. This effect, removal of associated RNase is also shown in Fig. 3. In two experiments with RLV, and others with SSV-1 (not shown), the yield of ^3H -labeled DNA:RNA hybrid was greater using the salt-treated preparation.

SDS polyacrylamide gel comparisons of treated and nontreated RD virus are shown in Fig. 4. The salt treatment resulted in a 25% loss of protein in these samples. However, there was a noticeable increase in the amount of viral proteins (p27, gp70) relative to the amount of total protein applied to the gels. Similar observations (not shown) were made with SSV-1, although the difference was less dramatic due, probably, to a smaller (12.5%) protein loss. As shown by these gels, no single protein appeared to be lost as a result of the process; however, the

amounts of those components identifiable as viral were often enhanced.

DISCUSSION

Sucrose gradient centrifugation is an effective means of concentrating and purifying tissue-cultured oncornaviruses. This method has not been absolutely effective in removal of non-viral proteins. Herein we have described a simple technique, using high concentrations of NaCl, that removes considerable amounts of contaminating materials left after gradient purification.

Some of these nonviral contaminants we have identified as components of FCS and exogenous (cellular) RNase. Since these were not removed by gradient centrifugation we presume that they are adsorbed (possibly by charge attraction) to the surface of the virions or to subcellular debris of the same density. The high concentration of NaCl facilitates desorption of these proteins, probably by interference with the aforementioned charge attraction. Once the proteins are desorbed the virus can be readily separated by high-speed centrifugation. This may be analogous to the disruption of

antigen-antibody complexes by NaCl as a means of antigen or antibody purification (7).

Salt treatment differs from the use of

TABLE 3. Reverse transcriptase specific activity of RD virus before and after salt treatment

Exp ^a	Treatment	Sp act ^b
I	None	19.7
	15% NaCl	67.5
II	None	8.2
	15% NaCl	12.9
III	None	30.1
	15% NaCl	90.9
IV	None	2.7
	15% NaCl	15.0

^a Data presented from four separate lots of virus salt or sham treated prior to assaying.

^b Specific activity expressed as picomoles of ³H-TTP incorporated into DNA per hour per milligram of protein in a standard reverse transcriptase assay.

TABLE 4. Effect of salt treatment on oncornavirus RNase activity

Virus	Treatment ^a	RNase activity (counts/min)
GALV-NC-37	None	767
	15% NaCl	188
RLV-V9 (6-day harvest)	None	1,172
	15% NaCl	511
RLV (2-day harvest)	None	227
	15% NaCl	291

^a Virus suspended in TNE or TNE-15% NaCl, incubated at 4 C for 30 min, and centrifuged prior to assessment for RNase activity as described in text.

TABLE 2. Effect of high NaCl treatment on infectivity

Virus and lot	Focus forming units/ml	
	Treated	Control
RLV ^a -230	10 ^{5.53}	10 ^{4.43}
RLV-234	10 ^{6.75}	10 ^{6.85}
RLV-235	10 ^{6.90}	10 ^{6.96}
SSV-100 ^b	10 ^{4.0}	10 ^{5.0}
SSV-102	10 ^{4.0}	10 ^{4.0}

^a Assayed on D-56 cells.

^b Assayed on rat normal kidney cells.

FIG. 2. Microimmunodiffusion results with salt-treated and control RD virus tested against (a) antiserum to ether-disrupted RD, (b) antiserum to FCS and (c) antiserum to salt-treated, ether-disrupted RD. (A) Ether-disrupted RD; (B) salt-treated, ether-disrupted RD; (C, D, E) FCS diluted 1:2, 1:5, or 1:10; (F) dialyzed, concentrated supernatant from salt treatment.

FIG. 3. Effect of salt treatment on reverse transcriptase activity. In two representative experiments, I and II, reaction products of twofold assays of RLV were examined by polyacrylamide gel analysis as described in the text. Results compare the effect of pretreatment of the virus with TNE + 15% NaCl (x) versus TNE alone (●).

(NH₄)₂SO₄, MeOH, or polyethylene glycol in that these reagents precipitate the virus from suspension. Other data, not reported here, reveal that a considerable amount of nonviral material can also be precipitated by these reagents. Using the NaCl method the virus is not precipitated, it is merely freed of adsorbed contaminants to allow subsequent concentration of the virus by high-speed centrifugation, leaving the (smaller) contaminants in the supernatant.

The initial function of salt treatment was to remove nonviral antigens prior to the preparation of hyperimmune antiviral sera. It was found that by salt treatment, the prime contaminants, FCS proteins, were reduced to the point that antibodies of those specifics were markedly reduced in the resulting antisera. The treatment did not appear to affect the content or immunogenicity of the viral proteins, as good antiviral activity was found in the antisera,

and antigens of treated and control virus compared favorably. Complement fixation data, not presented, have shown no evidence of adverse effects as a result of salt treatment. Titers of ether-disrupted RLV viral antigens were not reduced if the virus was treated prior to ether disruption.

It was of subsequent interest to learn if salt treatment had any adverse effects on other characteristics of these oncornaviruses. Analyses of the infectivity or biological activity of treated viruses showed no alteration as a result of treatment when compared to parallel controls. Those minor differences observed were determined to be within the range of experimental error. SDS acrylamide analyses showed also that viral proteins were not lost or detectably altered by the process. In some instances, where considerable protein loss was observed, there appeared to be an enhancement of amount of viral proteins (especially p27 and gp70) relative to the total protein. This, however, is attributed to a probable increase in the percentage of viral protein relative to the total protein.

The reverse transcriptase activity and the physicochemical characteristics of the nucleic acid are important characteristics for study of oncornaviruses. Salt treatment enhances the specific reverse transcriptase activity of treated viruses indicating that extraneous (inactive) protein has been removed. The increased activity, however, may be linked to the removal of exogenous nucleases by treatment. Once the nuclease is removed the template RNA is not degraded. It is possible that this may result in higher apparent specific activity. The higher yield of ³H-labeled DNA:RNA product—particularly of the 60 to 70S species (Fig. 3)—is important because it is seen that the high-molecular-weight RNA is not degraded by the salt treatment. Of equal importance is the indirect indication that this treatment may allow slightly more 60 to 70S specific ³H-labeled DNA product to be obtained from a given sample of virus for subsequent use in molecular hybridization studies.

It is of interest that, of the viruses studied here, only RD showed any morphological alterations. We consider the increased pleomorphism of RD to be of the minor significance in view of the naturally pleomorphic state of the virus under negative stain conditions (4).

Although we have applied the salt-treatment process to purification of oncornaviruses, we believe that it could be applicable to the purification of other viruses, microbial agents, or certain subcellular components.

FIG. 4. Comparison of control (B) and salt-treated (C) RD virus by SDS-acrylamide gel electrophoresis. Virus was disrupted by freeze thawing and sonic treatment; 100 μ g of protein was applied to each gel. (A) Protein standards: (1) phosphorylase A; (2) bovine serum albumin; (4) α -chymotrypsinogen; (5) cytochrome c.

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LITERATURE CITED

1. Aaronson, S. 1973. Biologic characterization of mammalian cells transformed by a primate sarcoma virus. *Virology* 52:562-567.
2. Bassin, R. H., N. Tuttle, and P. J. Fischinger. 1971. Rapid cell culture assay technique for murine leukemia viruses. *Nature (London)* 229:564-566.
3. Crowle, A. J. 1961. Immunodiffusion, p. 22. Academic Press Inc., New York.
4. Dessev, G. N., and K. Granchanov. 1973. Precipitation of RNA, DNA and nucleoprotein particles from very dilute solutions. *Anal. Biochem.* 53:269-271.
5. Furlong, N. B. 1967. DNA polymerase, p. 27-45. In H. Busch (ed.), *Methods in cancer research*, vol. III. Academic Press Inc., New York.
6. Harewood, K., and J. S. Wolff III. 1973. A rapid electrophoretic procedure for the detection of SDS-released oncornavirus RNA using polyacrylamide-agarose gels. *Anal. Biochem.* 55:573-581.
7. Landsteiner, K. 1962. The specificity of serological reactions, p. 137. Dover Publications, New York.
8. Lowry, O. H., N. J. Rosebrough, L. Farr, and R. J. Randall. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193:265-275.
9. Mayol, R., and R. Sinsheimer. 1970. Process of infection with bacteriophage ϕ X174. XXXVI. Measurement of virus specific proteins during a normal cycle of infection. *J. Virol.* 6:310-319.
10. Monroe, J., and P. Brandt. 1970. A rapid semiquantitative method for screening large numbers of virus samples by negative staining electron microscopy. *Appl. Microbiol.* 20:259-262.
11. Nowinski, R., L. Old, E. Boyse, E. de Harven, and G. Geering. 1968. Group specific viral antigens in the milk and tissues of mice naturally infected with mammary tumor virus of Gross leukemia virus. *Virology* 34:617-629.
12. Schlom, J., and S. Spiegelman. 1974. Simultaneous detection of reverse transcriptase and high molecular weight RNA unique to oncogenic RNA viruses. *Science* 174:840-843.
13. Stephens, R., K. Traul, G. Lowry, I. Zelljadt, and S. Mayyasi. 1972. Differential morphology of the RD virus from the human rhabdomyosarcoma, RD 114B cell line demonstrated by negative staining electron microscopy. *Nature (London) New Biol.* 240:212-214.
14. Traul, K. A., S. A. Mayyasi, C. E. Garon, G. Schildlovsky, and L. M. Bulfone. 1972. Antigenic comparison of Rauscher murine leukemia virus cultivated in human embryo and mouse cells. *Proc. Soc. Exp. Biol. Med.* 139:10-14.
15. Wright, B. S., P. A. O'Brein, G. P. Shibley, S. A. Mayyasi, and J. C. Lasfargues. 1967. Infection of an established mouse bone marrow cell line (JLS-V9) with Rauscher and Moloney murine leukemia viruses. *Cancer Res.* 27:1672-1677.