Heterogeneity in the Phospholipid Content of Purified Rabies Virus (ERA Strain) Particles

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SUMMARY

When rabies virus (ERA strain) was purified by precipitation with zinc acetate, Sephadex filtration, and centrifuging in a sucrose density gradient, usually about half of the virus particles spontaneously lost a portion of their envelope phospholipids. Because of differences in buoyant density, partially delipidized virus particles (about 1.16 g./cm.³) can be separated from intact virus (about 1.4 g./cm.³) by centrifuging in a sucrose density gradient. High egg passage virus (FLURY strain) purified by the same procedure, did not exhibit this type of heterogeneity. The release of phospholipids from ERA strain virus particles did not cause a marked decrease in the infectivity of the virus, but the morphology of the virus envelope changed from bullet- to bag-shaped. The protein (glycoprotein) composition and the RNA contents of intact and partially delipidized virus forms were similar.

INTRODUCTION

Extracellular high egg passage (HEP) rabies virus particles (strain FLURY) were reported to be heterogeneous with respect to particle length and sedimentation rate (Sokol et al. 1968; Sokol, 1971). In addition, infectious PM or FLURY HEP rabies virus particles were found to be heterogeneous with respect to their buoyant density in CsCl solution (Neurath, Wiktor & Koprowski, 1966; Schneider, Horzinek & Matheka, 1971). We report here on another type of heterogeneity in the sedimentation properties of purified ERA strain virus particles propagated in BHK-21 cells. It will be shown that this heterogeneity is due to specific changes which occur during purification in the structure of the virus envelope of some of the virus particles.

METHODS

Cells and virus. BHK-21 cells were grown in 1 l. Blake bottles as described previously (Sokol et al. 1968). The W clone of the ERA strain of rabies virus (Kuwert et al. 1968) was used in all experiments.

Infection of the cell cultures and labelling of the virus. BHK-21 cells were infected with
I to 5 p.f.u. virus/cell. After 1 hr adsorption at 33 °, the inoculum was removed and the cell cultures were re-fed with 80 ml. of Eagle's minimum essential medium supplemented with 0·2% bovine serum albumin. For labelling of the virus, the medium contained 0·5 μc/ml. of a mixture of 13 [14C]-amino acids (average specific activity, 250 mc/m-mole; Schwarz/Mann, Orangeburg, N.Y.) 2·5 μc of a mixture of 16 [3H]-amino acids/ml. (average specific activity, 17·6 c/m-mole; Schwarz/Mann) 0·13 μc of [14C]-lysine/ml. (specific activity, 165 mc/m-mole; Schwarz/Mann), 0·2 μc [3H]-tryptophan/ml. (specific activity, 4·6 c/m-mole; Schwarz/Mann), 1·3 μc [3H]-L-fucose/ml. (specific activity, 920 c/m-mole, Amersham/Searle, Arlington Heights, Ill.), 0·13 μc of [14C]-uridine/ml. (specific activity, 17·6 c/m-mole; New England Nuclear, Boston, Mass.) or 10 μc [3P]-orthophosphate/ml. (carrier-free; New England Nuclear). In experiments in which the protein moiety of the virus was labelled, the concentrations of the unlabelled amino acids in the medium were reduced to one-tenth of the regular level. The cultures were incubated at 33 °. Extracellular virus was harvested approximately 72 hr after infection.

Buffer solutions. NT buffer was composed of 0·13 M-NaCl and 0·05 M-tris (pH 7·8). NTE buffer contained in addition, 0·001 M-EDTA. All sucrose density gradients were made in NTE buffer.

Purification of the virus. The standard virus purification scheme included precipitation of virus particles from the infective tissue culture fluid by zinc acetate, desalting on a Sephadex column, sedimentating by centrifuging at high-speed and centrifuging in a sucrose density gradient (Sokol et al. 1968). In other purification procedures, precipitation of the virus by zinc acetate was omitted or replaced by precipitation of the virus by ammonium sulphate (300 g./l. of infective tissue culture fluid) in the presence of gamma globulin (0·6 g./l.).

Fractionation of virus particles by centrifuging in sucrose density gradient. Virus was centrifuged in sucrose density gradient at 4 ° in the SW 25·1 head of a Spinco centrifuge.

Virus assay. Concentrations of infectious virus were determined by plaque assay as described previously (Sedwick & Wiktor, 1967). Samples from sucrose density gradients were diluted tenfold in tissue culture medium containing 2% calf serum and stored at -7 ° until titrated.

Preparation and electrophoresis of polypeptides. Rabies virus was dissociated into poly-peptides and fractionated by electrophoresis in polyacrylamide gel containing sodium dodecyl-sulfate (SDS) as reported previously (Sokol, Stanček & Koprowski, 1971).

Determination of radioactivity. Total and acid-precipitable radioactivity was determined as described in a previous communication (Sokol et al. 1969).

Electron microscopy. Virus preparations negatively stained with phosphotungstic acid (Sokol et al. 1969) were viewed in a Siemens Elmiskop.

Chemical analysis. Protein was determined according to the method of Lowry et al. (1951), with bovine serum albumin as a standard.

RESULTS

Heterogeneity in sedimentation properties of purified ERA strain rabies virus particles

When ERA strain virus particles, concentrated and partially purified by the standard procedure, were centrifuged for 90 min. at 24,500 rev./min. in a 10 to 50% (w/w) linear sucrose density, in most experiments the virus was recovered in two (Fig. 1), or occasionally three (Fig. 7) distinct bands. These were designated as F1 (form 1), F2 and F3, in the order of increasing distance from the top of the gradient. Approximately equal amounts of virus were usually recovered from F1 and F2 bands. The specific infectivity of F1 and F2 viruses
Heterogeneity of rabies virus particles

Fig. 1. Heterogeneity of purified extracellular ERA virus particles labelled with [14C]-lysine and [3H]-fucose, after centrifuging in a sucrose density gradient. Fractions collected from the bottom of the gradient were assayed for E2 (●●●, panel a), total [14C] (●●●, panel b), and [3H] (○○○, panel b) radioactivities and infectivity (○○○, panel a).

was similar (about $1 \times 10^{11}$ p.f.u./mg. protein) as was the RNA and glycoprotein (Sokol et al. 1971; György, Sheehan & Sokol, 1971) content, judged from the corresponding specific radioactivities (Fig. 1, 7). Since the occurrence of F3 virus was rare, further studies were limited to comparison of the properties of F1 and F2 ERA virus particles.

To determine whether the virus forms differing in sedimentation properties corresponded to genetically different variants of ERA strain rabies virus, three clones each of F2 (eighth fraction, i.e., at 9.8 ml., from the bottom of the gradient shown in Fig. 1) and F1 (seventeenth fraction, i.e., at 13.3 ml., from the bottom of the same gradient) viruses were selected from plaques formed in agarose-suspended BHK-13S cells and further propagated in BHK-21 cell cultures to prepare seed virus. Extracellular virus produced in BHK-21 cell cultures infected with the different virus clones and labelled with radioactive amino acids was then concentrated and fractionated by centrifuging in a sucrose density gradient, as described in the legend of Fig. 1. Purified preparations of each virus clone contained similar amounts of F1 and F2 virus particles. These results indicated that F1 and F2 viruses are not different genetic variants of ERA strain rabies virus.

The heterogeneity in sedimentation properties of ERA virus particles was not caused specifically by treatment of the virus with zinc acetate, because virus particles precipitated
Fig. 2. Equilibrium centrifugation of purified extracellular ERA virus particles, labelled with [3H]-tryptophan, in a sucrose density gradient. Fractions collected from the bottom of the tubes were assayed for sucrose concentration (●—●) and total radioactivity (○—○). The same concentrated virus preparation was shown to contain an approximately equal proportion of F1 and F2 virus particles by the fractionation technique described in the legend of Fig. 1.

Fig. 3. Rate zonal centrifugation in a sucrose density gradient of purified extracellular ERA virus particles, labelled with [3H]-uridine. Fractions collected from the bottom of the tube were then assayed for sucrose concentration (●—●) and acid-insoluble radioactivity (○—○). The same concentrated virus preparation was shown to contain an approximately equal proportion of F1 and F2 particles by the fractionation technique described in the legend of Fig. 1.
Fig. 4. Sedimentation properties and buoyant density of ERA virus particles from unconcentrated, infective tissue culture fluid. Panel a: unconcentrated, infective tissue culture fluid, containing \[^{32}P\]-phosphate-labelled ERA virus, was centrifuged in 6 to 27 % (w/w) linear gradient of sucrose at 22,000 rev./min. for 25 min. Fractions were collected from the bottom of the tube and assayed for acid-insoluble radioactivity (○—○) and sucrose content (●—●). Panel b: unconcentrated, infective tissue culture fluid, containing ERA virus labelled with \[^{14}C\]-amino acid mixture, was centrifuged in 10 to 50 % (w/w) linear gradient of sucrose at 24,000 rev./min. for 90 min. Panel c: unconcentrated, infective tissue culture fluid containing ERA virus labelled with \[^{14}C\]-amino acid mixture, was centrifuged in 10 to 50 % (w/w) linear gradient of sucrose and at 24,000 rev./min. for 5 hr. The bands of radioactive material in the middle portion of the gradients contained 80 to 90 % of input infectivity (1 × 10^8 to 8 × 10^8 p.f.u.). Both virus preparations used in these experiments, after having been purified by the standard procedure, were shown to contain approximately equal amounts of F1 and F2 viruses.
Fig. 5. Comparison of polypeptide composition of F1 and F2 ERA viruses by electrophoresis in SDS polyacrylamide gels. The proportions of the four major polypeptides of the two virus forms were: F1, 44.3% glycoprotein (GIP), 34.2% nucleocapsid protein (NCP), 10.2% envelope protein 2 (EP2), and 11.3% envelope protein 3 (EP3); F2, 48.3% GIP, 30.9% NCP, 9.7% EP2, and 11.1% EP3. (Panel a is reprinted, with permission, from Sokol et al. 1971.)

with ammonium sulphate or sedimented by centrifuging at high speed before further purification by the standard procedure, showed after the final purification step a sedimentation pattern in sucrose density gradients similar to that shown in Fig. 1.

The conditions used in these experiments for banding of the virus in the 10 to 50% sucrose density gradients (24,500 rev./min. for 90 min.) were such that it was not clear whether equilibrium was actually reached, i.e., whether the different virus forms were separated mainly on the basis of differences in sedimentation rate or in buoyant density. When virus preparations concentrated and purified by the standard procedure were centrifuged for 5 hr in a similar sucrose density gradient to equilibrium, the virus was regularly recovered in a single, sharp band at 1.160 to 1.165 g./cm.³ density (Fig. 2), i.e., at the same density at which F2 particles were recovered after centrifuging for 90 min. (Fig. 1). These results suggested that purified ERA virus particles were either heterogeneous in sedimentation rate and homogeneous in buoyant density or that by centrifuging for 5 hr to equilibrium all F1 virus particles were converted into the denser F2 virus particles. To distinguish between these two alternatives, the homogeneity with respect to sedimentation
rate (after centrifuging for 15 min. in a 8 to 24 % sucrose density gradient at 22,000 rev./min.) of purified ERA virus particles was investigated (Fig. 3). The virus sedimented in a single band which was slightly skewed towards the bottom of the gradient. The relatively low degree of heterogeneity in sedimentation rate of purified virus particles could not account for the bimodal distribution observed in Fig. 1. Moreover, F1 virus particles, isolated by the fractionation technique described in the legend of Fig. 1, exhibited a similar slight heterogeneity in sedimentation rate (not shown here). The results so far described favoured the explanation that F2 virus is formed from F1 virus particles during the manipulations involved in the purification and fractionation of the virus and that centrifuging at 24,500 rev./min. for 90 min. in a 10 to 50 % sucrose density gradient was sufficient to separate
the two virus forms on the basis of their different buoyant densities. When the time of
equilibrium centrifugation was prolonged to 5 hr, all F1 virus particles were converted to
F2 virus.

Infective tissue culture fluid contained only F1 ERA virus, the buoyant density of which
is 1.135 to 1.140 g./cm.3, and which sediments in a shallow sucrose density gradient at an
essentially uniform rate (Fig. 4). The greater stability of ERA virus particles which were not
exposed to the treatments of the purification procedure, in comparison with purified virus,
was manifested by the lack of conversion of F1 into F2 virus even after centrifuging for 5 hr
in sucrose density gradient (Fig. 4, panel c).

Thus, conversion of F1 into F2 ERA virus is caused by manipulations used for rabies
virus purification. Since similar conversion of native virus particles into artificial virus
forms may pose a problem in purification or fractionation of several other enveloped
viruses, we decided to make a closer comparison of the properties of F1 and F2 ERA viruses,
in the hope of elucidating the reasons for this conversion.

Comparison of the chemical composition of F1 and F2 strain virus particles

Electrophoresis of dissociated F1 and F2 ERA viruses in polyacrylamide gel containing SDS
(Sokol et al. 1971) revealed that the two virus forms had similar polypeptide compositions,
the differences in the relative proportions of individual components being within the range
of experimental error (Fig. 5). Thus, conversion of F1 into F2 virus does not involve
specific or preferential loss of any of the four major virus proteins, which would be de-
tectable by this technique.

As mentioned above, the similarity in specific radioactivity of [3H]-uridine-labelled F1
and F2 viruses indicated that they contain similar amounts of RNA. The RNA from both
virus forms sedimented in sucrose density gradients at 45S (Fig. 6) corresponding to
undegraded virus RNA (Sokol et al. 1969).

To determine whether loss of lipids from the virus envelope was responsible for the
conversion of F1 virus into the denser virus forms, purified and [32P]-orthophosphate- and
[3H]-uridine-labelled ERA virus was fractionated by centrifugation in a sucrose density
gradient (Fig. 7). Labelled orthophosphate is primarily incorporated into the phospholipids
of the virus envelope, the amount of isotope incorporated into the virus RNA being
negligible by comparison (György et al. 1971). Thus, the [32P/3H] radioactivity ratio can
be considered an appropriate measure of the phospholipid-to-RNA ratio in the virus
particles. From the [32P/3H] ratios in the bands of the three virus forms, one can estimate
that F2 and F3 viruses contain 17 and 37%, respectively, less phospholipids than F1
virus. Therefore, the conversion of F1 ERA strain virus to F2 or F3 viruses is essentially
carried by the loss of a specific amount of phospholipids, and possibly of other lipids,
from the virus envelope.

Morphology of F1 and F2 ERA viruses

Electron microscopic examination of negatively stained preparations revealed marked
differences in the morphology of F1 and F2 viruses (Fig. 8). Despite the distortion of the
virus structure caused by the osmotic shock incurred during the removal of sucrose from
the samples of purified virus (Sokol et al. 1968), the overwhelming majority of F1 virus
particles were bullet-shaped, whereas most of the F2 virus particles had a bag-like shape.
Obviously, the loss of a part of the envelope lipids during conversion of F1 into F2 virus,
was accompanied by a collapse of the bullet-shaped virus shell. F2 virus particles seemed
to be more sensitive than F1 particles to the effect of osmotic shock, since nucleocapsids
were seen extruding from most of the former, but only a few of the latter.
Fig. 8. Morphology of negatively-stained F₁ (panel a) and F₂ (panel b) ERA rabies viruses. Arrows indicate the regions of the electron-micrographs enlarged in the insets.
DISCUSSION

The results of the present study are compatible with the following explanation of conversion of native ERA strain rabies virus forms during their purification. Fresh infective tissue culture fluids contain only one type (FI) of ERA virus particles. Loss of phospholipids and possibly of other lipids, from the virus envelope, which probably occurs in at least two distinct steps, causes the conversion of FI into F2, or F3 virus. The conversion into F3 ERA virus is a rare event and, therefore, the properties of F3 virus were not investigated in detail. Release of a part of the phospholipids from the envelope of FI virus particles effects a profound alteration in the shape of the virus particles, but does not significantly decrease their infectivity. This observation underlines the function of phospholipids in determining the shape of the virus envelope, but indicates that envelope phospholipids are not of primary importance for the uptake of the virus by host cells. Native ERA virus is occasionally converted, probably by further delipidization of intermediate F2 virus particles into F3 virus, the infectivity of which is markedly lower than that of FI virus. The finding that FI and F2 virus particles differ in buoyant density, but not in sedimentation rate, is not surprising. Rabies virus delipidized by treatment with the nonionic detergent Nonidet P-40 sediments at a rate similar to that of untreated virus (György et al. 1971), whereas its buoyant density is markedly higher than that of intact virus. Obviously the decrease in the particle weight of treated virus, which should result in decreased rate of sedimentation, is fully compensated for by a rise in its density and possibly by changes in shape, which tend to increase the sedimentation rate.

The reason for the delipidization of ERA strain virus particles during purification remains unknown. None of the steps in the standard purification procedure was found to be specifically responsible for the formation of artificial virus forms. The lability of the binding of phospholipids to other constituents of the virus envelope seems to be a unique property of the ERA rabies virus strain. Purified particles of FLURY HEP and PM strains do not lose phospholipids from their envelope during the standard purification procedure (Sokol et al. 1968), although the possibility of such loss under conditions of prolonged equilibrium centrifugation has not been investigated.

Our data on the heterogeneity of purified ERA rabies virus particles indicate that any heterogeneity in sedimentation properties of enveloped, lipid-containing viruses should be interpreted cautiously, bearing in mind the possibility that virus forms partially degraded during purification or fractionation might have retained a considerable part of the infectivity of intact virus. Similar caution should be exercised when extensively purified, enveloped viruses are analyzed for lipid content and composition.

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REFERENCES


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