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Release of Envelope Glycoprotein from Rabies Virions by a Nonionic Detergent

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Treatment of rabies virus with the nonionic detergent Nonidet P-40 resulted in solubilization of viral lipids and in a preferential release of the envelope glycoprotein. The other viral proteins and the viral ribonucleic acid remained associated in "core" particles sedimenting at a rate similar to that of intact virions. After fractionation of treated virus by velocity centrifugation in a sucrose density gradient, the amount of residual glycoprotein recovered in the "core" particle fraction and the extent of contamination of the glycoprotein fraction by other viral components were dependent on the ratio of detergent to viral protein used.

Rabies virions contain four major polypeptides of different molecular size. One of them constitutes the protein moiety of the viral nucleocapsid, whereas the remaining three are considered to be components of the viral envelope. The envelope component with a molecular weight of approximately 80,000 daltons has been shown to be a glycoprotein. It has been estimated that the envelope of a rabies virion contains 1,800 molecules of this glycoprotein (12).

The present report describes attempts to solubilize specifically the envelope glycoprotein of rabies virus. It will be shown that, at an appropriate ratio of detergent to virus, treatment of purified rabies virus with the nonionic detergent Nonidet P-40 (NP-40) solubilizes the viral phospholipids and results in a preferential release of the envelope glycoprotein. The bulk of the other protein components and the viral ribonucleic acid (RNA) remain associated in the viral "core" particles.

MATERIALS AND METHODS

Virus. The W clone of the ERA strain (7, 12) of rabies virus was used in all experiments.

Infection of the cells and labeling of the virus. BHK-21 cells were grown in 1-liter Blake bottles and infected with the virus as described previously (10). After 1 hr of adsorption at 35 C, the inoculum was removed, and each culture was refed with 70 ml of Eagle's minimum essential medium supplemented with 0.2% bovine serum albumin (BSA) and containing different combinations and amounts of radioactive compounds. Carrier-free ^{32}P-orthophosphate (10 μCi/ml; New England Nuclear Corp., Boston, Mass.) was used for labeling of the viral phospholipids and viral RNA; ^{3}H-uridine (1.8 μCi/ml; specific activity, 25 Ci/mmole; New England Nuclear Corp.) was used for specific labeling of the viral RNA; ^{3}H-D-glucosamine (1.6 μCi/ml; specific activity, 3 Ci/mmole; New England Nuclear Corp.) or ^{3}H-L-fucose (1.25 μCi/ml; specific activity, 920 mCi/mmole; Amersham Searle Corp., Des Plaines, Ill.) was used for labeling of the viral glycoprotein; and a mixture of 13 ^{14}C-amino acids (0.7 μCi/ml; average specific activity, 250 μCi/mmole; Schwarz/Mann mixture no. 3122-08, Schwarz BioResearch, Orangeburg, N.Y.) or ^{14}C-lysine (0.13 μCi/ml; specific activity, 165 mCi/mmole; Schwarz BioResearch) was used for labeling of viral proteins. For labeling of proteins with ^{14}C-amino acids, the concentration of cold amino acids in the medium was reduced to 10% of the normal level. The cultures were incubated at 33 C. Extracellular virus was harvested approximately 72 hr after infection.

Purification of the virus. The virus purification scheme included precipitation by zinc acetate, desalting on a Sephadex column, pelleting by high-speed centrifugation, and banding of the virus in a sucrose density gradient (10). ERA virions are heterogeneous with respect to phospholipid content and are recovered after centrifugation in a sucrose density gradient (10) in two or three distinct bands (F. Sokol, H. F. Clark, and E. György, *manuscript in preparation*). Only virions with the highest lipid content were used in the present study. They were collected through a puncture in the side of the tube, diluted with buffer solution, repelleted by high-speed centrifugation, and dissolved in 0.13 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.001 M ethylenediaminetetraacetate, *p*H 7.8 (NTE buffer). The virus was stored at 4 C for no longer than 48 hr before being treated with NP-40.

Protein determination. Protein was assayed as described by Lowry et al. (8) with BSA used as standard.

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Preparation and electrophoretic fractionation of polypeptides. Dissociation of virions or their components into polypeptides and their fractionation by electrophoresis in a polyacrylamide gel, containing sodium dodecyl sulfate, were described previously (12).

Determination of radioactivity. Total and acid-precipitable radioactivity were determined as described before (11). When desired, ^{32}P-phosphate labeled phospholipids were removed from acid-insoluble precipitates by repeated washing with acetone at 0 C. The determination of radioactivity contained in gel slices was also described previously (12).

RESULTS

Solubilization of the envelope glycoprotein and of phospholipids by treatment of rabies virus with NP-40. Virus labeled with ^{3}H-glucosamine and ^{14}C-amino acid was treated at 22 C for 20 min with increasing amounts of NP-40 (Shell Chemical Co., Chicago, Ill.), and the treated virions were then fractionated by velocity centrifugation in a sucrose density gradient. The viral glycoprotein was preferentially solubilized at detergent

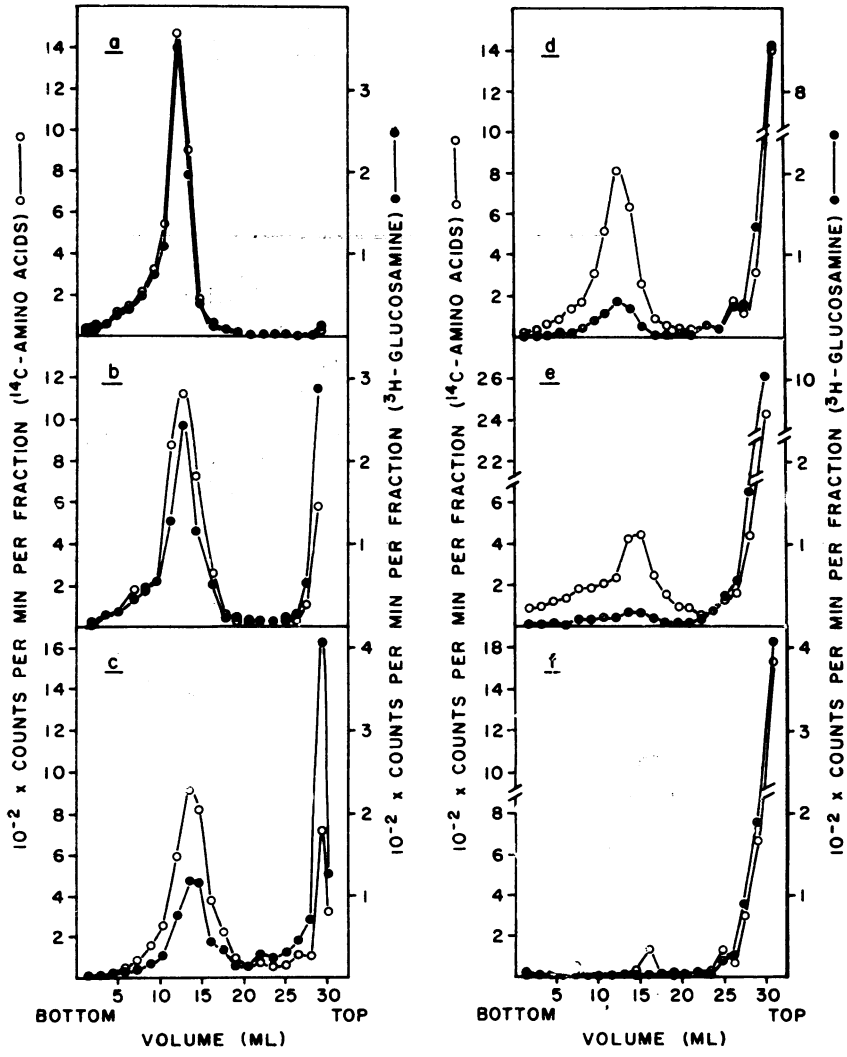

FIG. 1. *Fractionation by velocity centrifugation in a sucrose density gradient of rabies virus treated with different amounts of Nonidet P-40 (NP-40). Samples of purified virus suspension labeled with ^{3}H-glucosamine and ^{14}C-amino acid, each containing 23 μg of viral protein in 0.05 ml of NaCl-Tris-EDTA (NTE) buffer, were mixed with 0.025 ml of 0.15% (b), 0.6% (c), 3.0% (d), 15.0% (e), or 30.0% (v/v) (f) NP-40 in distilled water and kept at 22 C for 20 min. Virus treated similarly with distilled water (a) served as a control sample. Each mixture was then layered over 28 ml of a linear 5 to 23% (w/w) gradient of sucrose in NTE buffer. The gradients were centrifuged for 25 min at 51,000 × g, at 4 C, in a Spinco SW 25.1 rotor. Acid-precipitable radioactivity in fractions collected from the bottom of the gradient was then determined.*

to viral protein ratios (milliliters of NP-40 per gram of protein) equal to or lower than 33 (Fig. 1). Released glycolipids could also contain a small portion of radioactive carbohydrate found in the top fractions of the gradient. D-Glucosamine, however, is not a constituent of glycolipid contained in BHK cells or in envelopes of viruses grown in these cells (5, 6). Moreover, solubilized glycolipids are acid-soluble. Thus, the acid-insoluble ^{3}H radioactivity recovered from fractions at the top of the gradient represents glucosamine incorporated into the viral glycoprotein. At detergent to protein ratios of 165 or higher, the specificity of the action of NP-40 in solubilizing the viral glycoprotein seems to be decreased. This was indicated by the observation that at an NP-40 to protein ratio of 165, about 55% of the ^{14}C-amino acid label became solubilized, whereas the viral glycoprotein represents only about 44% of the total viral proteins (12). Essentially all viral proteins became solubilized at a detergent to protein ratio of 330.

The sedimentation coefficient of the viral "core" particles, recovered in the middle portion of the gradient, was only slightly lower than that of untreated virus. On the other hand, their buoyant density in sucrose solution was appreciably higher than that of intact virions (Fig. 2). The amount of residual glycoprotein in the "core" particles decreased with the increasing amount of detergent used for the treatment. The relative proportions of acid-insoluble ^{3}H-glucosamine and ^{14}C-amino acid radioactivity recovered in the "core" particle band and at the top of the gradient, after fractionation of virions treated with various amounts of detergent, are summarized in Fig. 3. This relationship can be used for

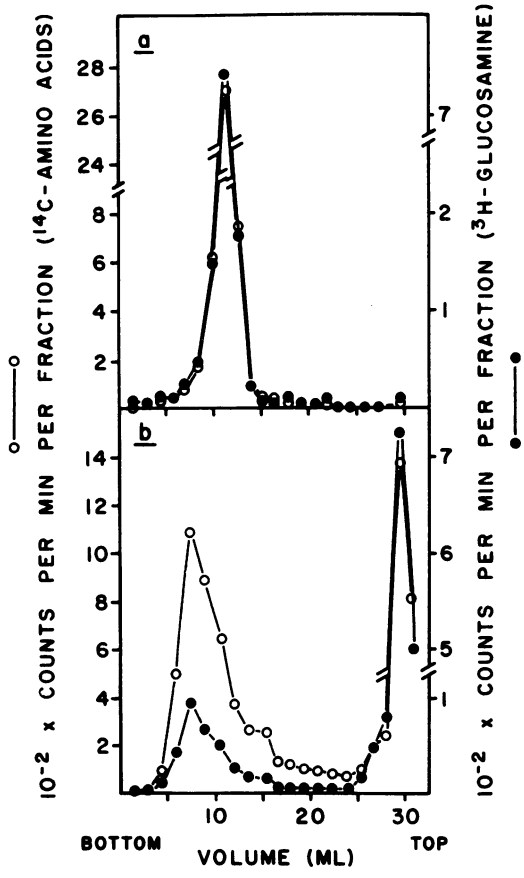

FIG. 2. *Fractionation by equilibrium centrifugation in a sucrose density gradient of untreated and Nonidet P-40(NP-40)-treated rabies virus. Two samples of purified virus suspensions labeled with ^{3}H-glucosamine and ^{14}C-amino acid, each containing 23 μg of viral protein in 0.05 ml of NaCl-Tris-EDTA (NTE) buffer, were mixed with 0.025 ml of distilled water (a) or with the same volume of 3% NP-40 in distilled water (b). After being kept at 22 C for 20 min, the mixtures were layered over 28 ml of a linear 10 to 50% (w/w) gradient of sucrose in NTE buffer. The gradients were centrifuged for 5 hr at 56,000 × g, at 4 C, in a Spinco SW 25.1 rotor. Acid-precipitable radioactivity in fractions collected from the bottom of the gradient was then determined. The buoyant density of untreated virus was 1.16 g/cm³, whereas that of the majority of viral "core" particles was found to be 1.20 g/cm³. Note the obvious heterogeneity of viral "core" particles with respect to their buoyant density. The densities of the top and bottom fractions in both gradients were 1.060 and 1.225 g/cm³, respectively.*

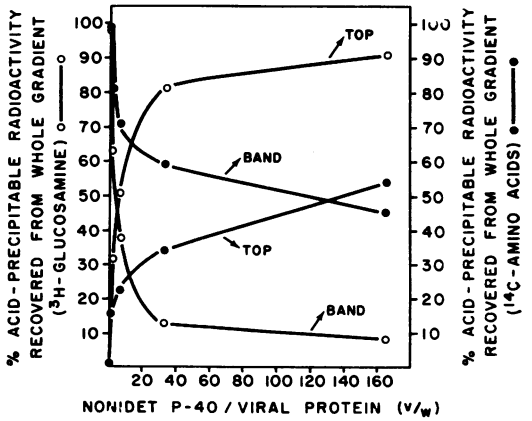

FIG. 3. *Relative proportions of solubilized and of "core" particle-associated glycoprotein and total viral protein in preparations of rabies virus treated with different amounts of Nonidet P-40. Percentages of the acid-insoluble radioactivity contained in whole gradients (see Fig. 1a, b, c, d, and e), which were recovered in the band of viral "core" particles or in two to four fractions at the top of the gradients, were plotted against the detergent to viral protein ratio. Proportions of radioactivity recovered in other fraction of the gradient (less than 7% of the total) are not shown.*

estimating the amount of NP-40 required for an almost quantitative and still highly selective release of viral glycoprotein from the rabies virus envelope.

Under conditions causing almost all viral glycoprotein to become solubilized by NP-40, the bulk of the acid-precipitable viral RNA remained associated with the "core" particles (Fig. 4). On the other hand, the phospholipids of the viral envelope were solubilized by the detergent, as indicated by the distribution of radioactivity after centrifugation of ^{32}P-phosphate-labeled and NP-40-treated virions in a sucrose density gradient (Fig. 5). The relatively small amount of ^{32}P-radioactivity recovered in the "core" particle band most likely represents incorporation of the radioactive phosphate into the viral RNA. The bulk of acid-insoluble ^{32}P radioactivity which could not be removed from the fractions at the top of the gradient, even by repeated washing with acetone, might correspond to phosphorylated proteins. A phosphoglycoprotein, phosvitin, was reported to be contained in hen's egg yolk (9).

When a detergent to viral protein ratio of 33 was maintained, the variation of factors, such as final NP-40 concentration [0.1 to 1% (v/v)], time of treatment (20 to 60 min), and temperature (4 to 22 C), did not essentially affect the extent of decomposition of the rabies virions.

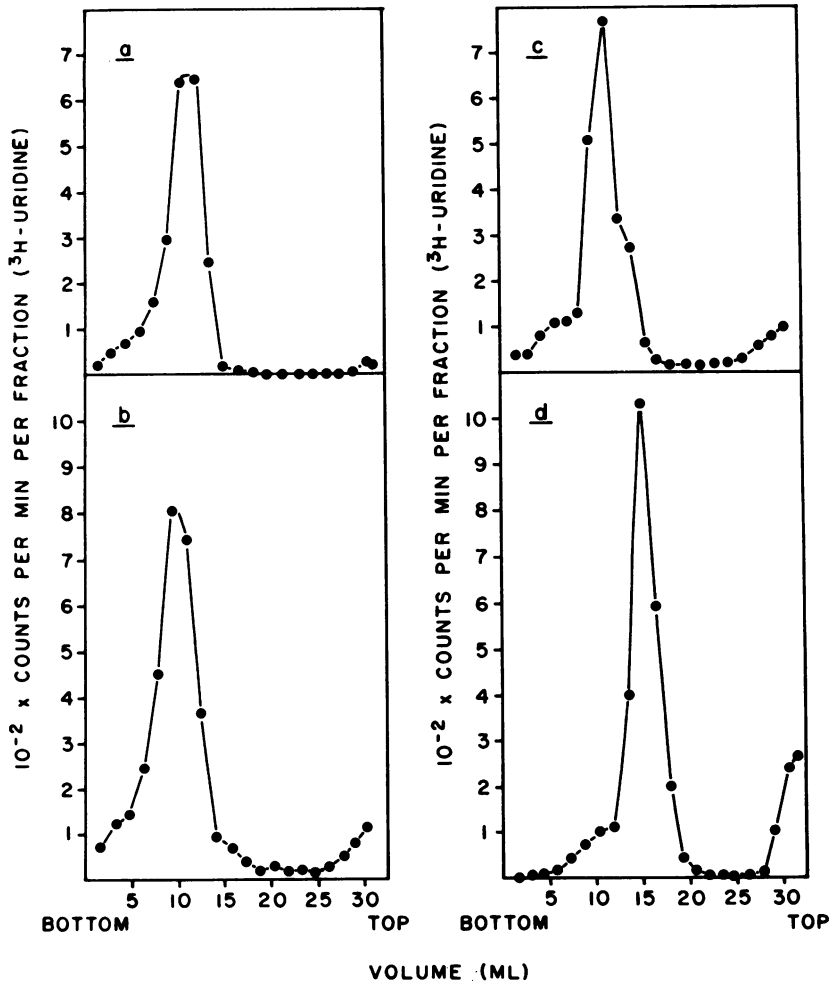

FIG. 4. *Lack of release by Nonidet P-40 (NP-40) of viral RNA from rabies virions. Samples of purified virus suspension labeled with ^{3}H-uridine, each containing 23 μg of protein in 0.05 ml of NaCl-Tris-EDTA buffer, were mixed with 0.025 ml of 0.15% (b), 0.6% (c), or 3.0% (d) NP-40 in distilled water and kept at 22 C for 20 min. Virus treated similarly with distilled water (a) served as a control sample. Samples were then fractionated by velocity centrifugation in a sucrose density gradient and analyzed as described for Fig. 1. Relative proportions of acid-precipitable ^{3}H-uridine recovered at the top of the gradients: 2% (a), 7.9% (b), 9.4% (c), or 19.3% (d) of the radioactivity recovered in the corresponding whole gradient.*

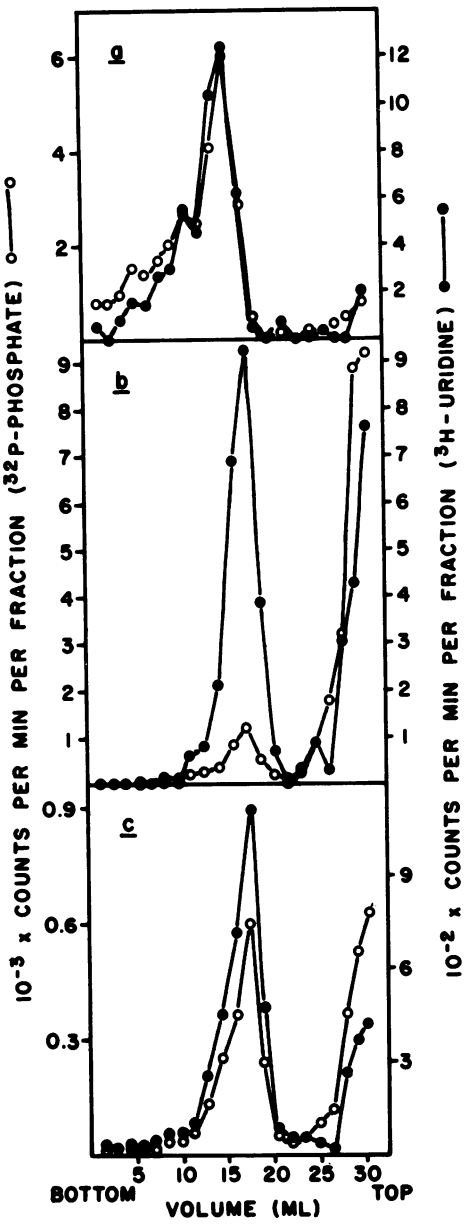

FIG. 5. *Solubilization by Nonidet P-40 (NP-40) of phospholipids from rabies virus envelope. Samples of purified virus labeled with ^{32}P-phosphate and ^{3}H-uridine, each containing 21 μg of viral protein in 0.05 ml of NaCl-Tris-EDTA buffer, were mixed with 0.025 ml of distilled water (a) or with the same volume of 3% NP-40 (b and c) in distilled water and kept at 22 C for 20 min. Samples were then fractionated by velocity centrifugation in a sucrose density gradient as described for Fig. 1. In fractions of untreated virus, acid-precipitable radioactivity was determined (a). In fractions of treated virus, both total (b) and acid-precipitable (c) radioactivities were determined. Acid-insoluble precipitates were washed twice with acetone at 0 C to remove contaminating phospholipids.*

Electrophoretic analysis of glycoprotein and "core" particle fractions. The tentative conclusions, outlined above, concerning the specificity of NP-40 action on rabies virus were confirmed by data obtained from electrophoretic analysis of the polypeptide composition of the viral "core" particles and of proteins released by the nonionic detergent (Fig. 6). At NP-40 to protein ratios of 66 or lower, more than 75% of released proteins represented envelope glycoprotein, whereas the "core" particles still contained an appreciable amount of residual glycoprotein. When the detergent to viral protein ratio was higher than 66, the glycoprotein content of the "core" particles was markedly decreased.

Results of experiments carried out with ^{3}H-L-fucose-labeled virus show that this sugar, like D-glucosamine (12), is a constituent of rabies virus glycoprotein.

DISCUSSION

We showed previously that treatment of rabies virus with sodium deoxycholate (DOC) results in release of the viral nucleocapsid and solubilization of the viral envelope. The nucleocapsid can be separated from the envelope components by velocity centrifugation in a sucrose density gradient (11). Attempts to fractionate the mixture of the three envelope proteins further (12) by isoelectric focusing or by ion-exchange chromotography (D. Stanček and F. Sokol, *unpublished data*) were largely unsuccessful because a considerable part of the ionic detergent remained bound to the envelope components and could not be removed from the mixture even by exhaustive dialysis. The fact that the nondialyzable portion of DOC occasionally gelled and that removal of solubilized viral lipids by extraction of the mixture with organic solvents resulted in denaturation of envelope proteins presented additional difficulties. We decided, therefore, to search for other methods for solubilization of the rabies virus envelope.

Cartwright et al. (1, 2) reported that treatment of vesicular stomatitis virus (VSV), which is structurally similar to rabies virus, with NP-40 released the immunizing antigen from the envelope, leaving behind a "skeleton" similar in size and shape to the intact virion. The "skeleton" particles lack the lipid envelope and the surface projections but contain the viral nucleocapsid and the other envelope proteins. It was shown recently that the immunizing antigen of VSV is identical to the glycoprotein component of this virus (4). Results similar to those described for VSV were obtained in the present study on the effect of NP-40 on rabies virions. Rabies virus glycoprotein is preferentially, but not exclusively, released by the detergent from the viral envelope. To suppress contamination of the solubilized glycopro-

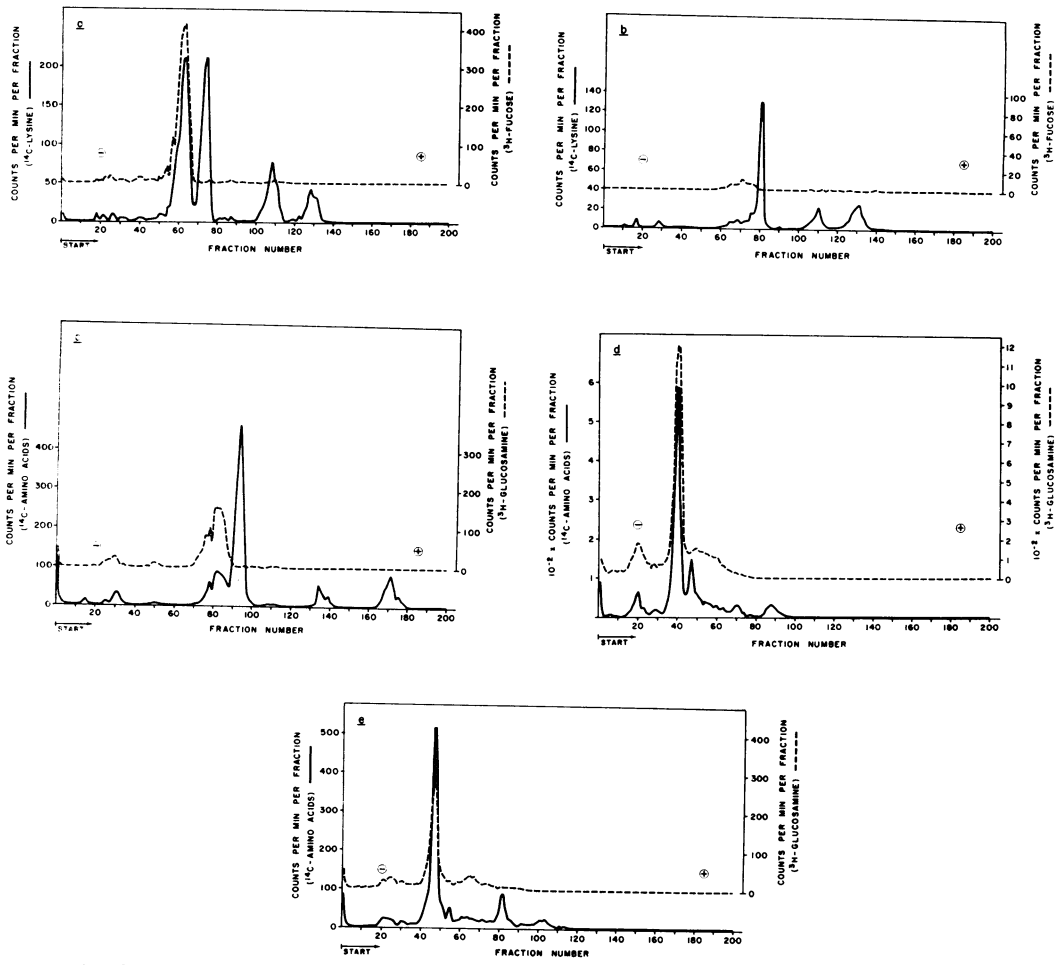

FIG. 6. *Electrophoretograms of polypeptides derived from glycoprotein and "core" particle fractions of Nonidet P-40 (NP-40)-treated rabies virus. Purified rabies virus was treated with the detergent at 22 C for 20 min and then fractionated by velocity centrifugation in a sucrose density gradient as described for Fig. 1. "Core" particles (two to three pooled fractions from the middle portion of the gradient) and the crude envelope glycoprotein (two top fractions of the gradient) were dissociated by sodium dodecyl sulfate in a reducing environment and subjected to electrophoresis in a 7.0% polyacrylamide gel (12). (a) Rabies virus labeled with ^{14}C-lysine and ^{3}H-fucose which was not treated with NP-40. Electrophoresis was for 15 hr at 5 ma/gel. The four major viral polypeptides, in order of decreasing size, are: envelope glycoprotein, nucleocapsid protein, envelope protein 1, and envelope protein 2 (12). (b) "Core" particles isolated from ^{14}C-lysine- and ^{3}H-fucose-labeled and NP-40-treated virus (detergent to viral protein ratio, 165). Electrophoresis was for 17 hr. Residual glycoprotein represents about 12% of total polypeptides. (c) "Core" particles isolated from ^{14}C-amino acid- and ^{3}H-glucosamine-labeled and NP-40-treated virus (detergent to viral protein ratio, 33). Electrophoresis was for 19 hr. Residual glycoprotein represents about 17% of total polypeptides. (d) Crude glycoprotein isolated from ^{14}C-amino acid- and ^{3}H-glucosamine-labeled and NP-40-treated virus (detergent to viral protein ratio, 33). Electrophoresis was for 11 hr. About 87% of the polypeptides correspond to glycoproteins. (e) Crude glycoprotein isolated from ^{14}C-amino acid- and ^{3}H-glucosamine-labeled and NP-40-treated virus (detergent to viral protein ratio, 66). Electrophoresis was for 12 hr. About 76% of the polypeptides correspond to glycoproteins.*

tein fraction with other viral proteins, the virions have to be treated with amounts of detergent insufficient for dissociation of all of the glycoprotein from the viral "core" ("skeleton") particles. On the other hand, "core" particles essentially free from glycoprotein can be obtained only after treatment of rabies virus with relatively large amounts of NP-40, which also effect partial solubilization of protein components other than the envelope glycoprotein. Thus, the detergent to

viral protein ratio should be varied, depending on whether the purity of the glycoprotein fraction or of the "core" particles is being emphasized. We did not observe aggregation of "core" particles derived from NP-40-treated rabies virions, as has been described for VSV "skeletons" (1); the sedimentation coefficient of rabies virus "cores" was similar to that of intact rabies virions.

The simple procedure described in the present study permits the fractionation of rabies virions into crude glycoprotein and "core" particle preparations. Experiments are now in progress to isolate the three envelope proteins of rabies virus in a pure and immunologically active form from these two fractions. Results of preliminary studies performed by other investigators have shown that the immunogenicity of rabies virus is essentially retained after treatment with NP-40 (3).

ACKNOWLEDGMENTS

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