Characterization of Subviral Components Resulting from Treatment of Rabies Virus with Tri(n-butyl) Phosphate

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SUMMARY

The PM strain of rabies virus was grown in human diploid WI-38 cells and labelled with the following radioactive substances: $[^{14}\text{C}]$- or $[^3\text{H}]$-$d$-glucosamine, $[^3\text{H}]$-uridine, $[^1\text{C}]$-stearic acid, $[^3\text{H}]$-choline and a mixture of $[^1\text{C}]$-amino acids. It was then purified and treated with 0.1% tri(n-butyl)phosphate (TNBP) in the presence of 0.1% Tween 80. All the stearic acid and choline and about 82% and 40%, respectively, of the $d$-glucosamine and amino acid (but none of the uridine) label was released from the virus. It was concluded that TNBP dissociates the lipids, most of the glycoproteins, and small quantities of non-glycosylated proteins from the virus leaving behind particles containing all of the virus RNA. These particles sedimented in sucrose gradients at the same rate as did intact virus; they had a higher buoyant density in CsCl gradients (1.304 compared with 1.244 $g./cm^3$) and they resembled intact virus in overall size and shape but lacked most of the surface projections. By polyacrylamide gel electrophoresis the following polypeptide species were identified in intact particles (tentative molecular weights are given in parentheses): glycoproteins GP1 (78,000) and GP2 (65,000); nucleocapsid proteins NP1 (58,000) and NP2 (47,000) and two additional proteins MP (35,000) and CP (22,000). Treatment with TNBP released nearly all GP1 and GP2 and small quantities of MP. Particles obtained by treatment of the virus with the proteolytic enzyme bromelain also lacked the surface projections (and the glycoproteins GP1 and GP2) but retained the lipids.

INTRODUCTION

The tissue culture medium, from BHK 21 or human diploid WI-38 cells infected with rabies virus, contains soluble virus-specific antigens, detectable by precipitin or complement fixation tests (Neurath, 1966; Wiktor et al. 1969). These elicit the formation of virus-neutralizing antibodies in immunized animals (Wiktor et al. 1969; Schlumberger, Wiktor & Koprowski, 1970; Crick & Brown, 1970; Dobkin et al. in preparation). Envelope antigens, obtained by disrupting purified rabies virus with detergents, also induce the formation of virus neutralizing antibodies (Crick & Brown, 1970; Sokol, Stanček & Koprowski, 1971). These results suggest that subviral components derived from rabies virus might become a useful and safe tool for vaccination against rabies.

The immunological properties of envelope antigens derived from influenza viruses greatly depend on the methods by which these antigens are prepared from intact viruses (Neurath &
Rubin, 1971). Recent results (Neurath et al. 1971) indicate that the method of choice in this respect consists of disrupting the viruses by tri-(n-butyl)phosphate (TNBP) in the presence of Tween 80. The same approach has led to the successful preparation of an experimental rabies vaccine (data to be published). The present study aims at the characterization of the subviral components resulting from treatment of rabies virus with TNBP. The immunological properties of these components will be described in detail elsewhere.

METHODS

Propagation of virus. The PITMAN-MORE (PM) strain of rabies virus, adapted to growth in human diploid WI-38 cells and propagated in these cells for 54 passages, was provided through the courtesy of Dr T. J. Wiktor of the Wistar Institute, Philadelphia, Pennsylvania. It was propagated essentially under conditions described elsewhere (Wiktor et al. 1969). The infected WI-38 cell cultures were transferred twice weekly, using trypsin to detach the cells from glass (Wiktor, Fernandes & Koprowski, 1964). After the second transfer, tissue culture medium supplemented with 0.5% human albumin ('maintenance medium') instead of 10% calf serum was used to facilitate the subsequent virus purification (Sokol et al. 1968). The fluids containing virus were harvested 48 hr after the second transfer of cells.

Purification of virus. The infectious tissue culture fluids, clarified by low-speed centrifugation, were precipitated with zinc acetate as described before (Neurath, Wiktor & Koprowski, 1966). The precipitate was dissolved in a saturated solution of EDTA, adjusted to pH 7.8 by addition of solid tris. The volume of the solution corresponded to 1/50 of the vol. of the tissue culture fluid. The concentrated virus material was centrifuged at 26,000 rev./min. for 1 hr in the SW65 rotor of an L4 preparative ultracentrifuge (Spinco Division, Beckman Instruments, Palo Alto, California). The pellets were suspended in a suitable volume of NTE buffer (0.13 M-NaCl, 0.05 M-tris chloride, 0.001 M-EDTA, pH 7.8; Sokol et al. 1968). The resulting suspension was clarified at 2000 rev./min. for 5 min. in an IEC centrifuge, size 2 (International Equipment Co., Boston, Massachusetts). Samples of 1 ml. were submitted to rate zonal centrifugation (21,000 rev./min. for 17 min., Spinco rotor SW25.1) in a sucrose gradient (28 ml.; 10 to 25% sucrose in NTE buffer containing 10 mg. of bovine albumin/100 ml.). About 18 fractions were collected from the bottom of the centrifuge tubes, using a peristaltic pump. The middle fractions, corresponding to the peak of radioactivity when isotopically labelled virus material was used (see Results), were pooled. For electron microscopy the pooled fractions were centrifuged for 1 hr at 26,000 rev./min. in the SW65 rotor and the pellets were resuspended in NTE buffer.

Radioactive labelling. For labelling the virus, one of the following radioactive substances was added to the maintenance medium at a level of 0.5 to 1.0 μc/ml.: d-glucosamine-1-[^14]C hydrochloride (59 mc/m-mole), d-glucosamine-6-[^3]H]-hydrochloride (3.6 c/m-mole), uridine-2-[^14]C)-(55.6 mc/m-mole), choline-methyl[^3]H]-chloride (0.55 c/m-mole) (all four from New England Nuclear, Boston, Massachusetts), stearic-1-[^14]C]-acid (4 mc/m-mole) and a mixture of [^14]C]-amino acids (algal hydrolysate; 1 mc/mg.) (both from International Chemical and Nuclear Corporation, Irvine, California). Infected and uninfected control cells were maintained in the presence of each of the labelled substances for a period of 48 hr. The labelled viruses were purified as described above.

Treatment with TNBP. In each instance, where not stated otherwise, 9 parts of a suspension of purified virus in NTE buffer containing 100 μg./ml. of bovine albumin was mixed with 1 part of a suspension containing 1% (v/v) each of Tween 80 and TNBP (Fisher Scientific Co., Fair Lawn, New Jersey). The mixture was incubated for 1 hr at 37° and subsequently
fractionated by rate zonal centrifugation under the conditions described for the purification of intact virus.

*Treatment with bromelain.* Purified virus, suspended in NTE buffer containing 0.01% mercaptoethanol, was treated with bromelain (13 mg./ml., Calbiochem) for 90 min. at 37°C. The treated virus was then submitted to rate zonal centrifugation (21,000 rev./min. for 17 min., rotor SW25.1). When [14C]-amino acid-labelled virus was centrifuged, two peaks of radioactivity appeared, one at the top of the gradient, and another about one-third of the total distance across the gradient (measured from the top). Fractions corresponding to the faster sedimenting peak were pooled and centrifuged in 4 ml. portions for 1 hr at 26,000 rev./min. in a Spinco rotor SW65. The pellets, resuspended in NTE buffer, were used for further studies.

*Isopycnic density-gradient centrifugation in CsCl gradients.* Samples (1.3 ml.) of a suspension of purified virus or of components resulting from the treatment of the virus with TNBP were layered over 3.5 ml. of a solution of CsCl (refractive index = 1.3644) in 0.01 M-tris HCl (pH 7.0) that had been placed into centrifuge tubes for the SW65 rotor. Centrifugation was performed at 35,000 rev./min. for 48 to 72 hr at 15°C. The refractive indexes of fractions collected from the bottoms of the tubes were determined using an Abbe-3L refractometer (Bausch & Lomb, Rochester, New York). The densities of the fractions were calculated according to equations given by Vinograd & Hearst (1962).

*Isolation of the nucleocapsid of rabies virus.* The viral nucleocapsid was isolated from sodium deoxycholate (DOC)-disrupted virus particles by rate zonal centrifugation in sucrose gradients under conditions similar to those described by Sokol et al. (1969).

*Measurement of radioactivity.* Suitable volumes of samples were placed into vials containing 20 ml. of scintillation fluid (100 g. of naphthalene, 10 g. of 2,5-diphenyloxazole, 250 ml. of 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene, 200 ml. of methanol and 1000 ml. of p-dioxane). Radioactivity was measure in a Packard 3375 Tri-carb liquid scintillation counter. The viral components in fractions obtained after rate zonal centrifugation were precipitated by adding 0.2 ml. of trichloracetic acid (TCA) (1 g./ml.). Bovine albumin (1 ml. of 0.01%) was added to fractions obtained after isopycnic centrifugation prior to the addition of TCA. All samples were then filtered through 0.45 μm. Millipore filters using filtering centrifuge tubes (Millipore Filter Corp., Bedford, Massachusetts). The filters with the radioactive precipitates were dissolved in the scintillation fluid.

*Electron microscopy.* Specimens were negatively stained with 0.5% ammonium molybdate and observed with an RCA EMU-3H electron microscope employing double condenser illumination at 50 kv.

*Tests for immunizing potency.* Mice were immunized with serial 5-fold dilutions of the intact or treated virus and subsequently challenged with live virus following the procedures described by Wiktor et al. (1969).

*Dissociation of virus proteins and electrophoresis on polyacrylamide gels.* The polypeptide composition of rabies virus and the subviral components derived from it was determined by the method described by Maizel, White & Scharff (1968). The purified virus or the ‘core’ particles obtained by treatment of the virus with TNBP were submitted to high-speed centrifugation. The resulting pellets were dissolved in 0.1 M-sodium phosphate buffer, pH 7.35 containing 2.5% (v/v) mercaptoethanol (ME) and 2.5% (w/v) sodium lauryl sulphate (SLS: specially pure, BDH Chemicals Ltd., Poole, England). Alternatively, fractions from sucrose gradients containing purified virus or subviral components were precipitated with TCA (Sokol et al. 1971) and subsequently redissolved in phosphate buffer containing 2.5% each of ME and SLS. The samples were incubated at 37°C for 1 hr and subsequently at 100°C.
for 1 min., then dialysed at room temperature overnight against phosphate buffer containing 0·1% each of ME and SLS.

Polyacrylamide gels were prepared in the following way: 0·2 g. of SLS, 16 g. of Cyanogum-41 (E-C Apparatus Corp., Philadelphia, Pennsylvania), 0·2 g. of ammonium persulphate and 0·2 ml. of \(N,N',N',N'\)-tetramethylethylenediamine were added, in that order, to 184 ml. of 0·025 M-sodium phosphate buffer, \(pH\) 7·35. The resulting solution was poured into the space between the cooling plates of an EC470 vertical gel electrophoresis cell and allowed to solidify for at least 30 min. Sodium phosphate buffer (0·025 M) containing 0·1% SLS was used as electrode buffer and was continuously circulated between the cathode and anode compartments of the electrophoresis cell. Tap water served as coolant for the cooling plates. The gel was pre-run for 1 hr at 200 v. The samples, to which sucrose had been added to a final concentration of about 15%, were applied on top of the slots in the gel. Four samples were run simultaneously in a single gel plate for 140 rain. at 250 v. After completion of the run, the gel was cut into 4 strips, each corresponding to one sample. The strips were further cut into slices \(\frac{1}{2}\) in. wide, each of which was placed into a scintillation vial. The polypeptides were digested and eluted from the gel by incubating the slices at 60° overnight in 1·3 ml. of a 0·1% solution of protease 62 (Rohm & Haas, Philadelphia, Pennsylvania) in 0·1 M-tris (no acid added for \(pH\) adjustment). Protease 62 has a temperature optimum between 50 and 60° and a \(pH\) optimum between 8 and 9. Scintillation fluid (20 ml.) was added to each of the vials and the radioactivity was measured as described above.

The following proteins served as standards for the determination of the molecular weights of the polypeptides of rabies virus: horse heart cytochrome c, sperm-whale myoglobin, ovalbumin, bovine serum albumin, bovine \(\gamma\)-globulin and lysozyme. These proteins were submitted to electrophoresis under the same conditions as described for rabies virus. The gels were fixed in 20% (w/v) sulphosalicylic acid and the position of the protein bands was revealed by staining with a 0·2% solution of Amido Black 10 B in 45% (v/v) methanol-9% (v/v) acetic acid. The excess of dye was removed by electrophoretic destaining using the EC489 destainer. The molecular weights were determined by the method of Shapiro, Viñuela & Maizel (1967).

**RESULTS**

**Purification of rabies virus**

The procedure described previously for the concentration and purification of extracellular rabies virus produced in hamster cells (Neurath et al. 1966; Sokol et al. 1968) was shown to be adequate to allow the biochemical and biophysical characterization of that virus (Sokol et al. 1968, 1969, 1970), and it seemed likely that a similar procedure would also be suitable to purify rabies virus grown in diploid WI-38 cells. The distribution of radioactivity throughout the sucrose gradients after centrifugation of the infected and uninfected \(^{14}\text{C}\)-amino-acid-labelled tissue culture material (Fig. 1) was in accordance with this expectation. Similar results were obtained with material labelled with the other radioactive substances. The purification of the virus also was confirmed by electron microscopy. Thus, the radioactive material harvested from the middle fractions, corresponding to the peak in Fig. 1 (and coinciding with the peak of infectivity as established by separate experiments), was considered suitable for investigating the fate of the virus upon its reaction with TNBP.

**Solubilization of virus lipids and phospholipids by treatment with TNBP**

The disruption of the envelopes of lipid containingviruses by lipid solvents and/or detergents may be ascribed to perturbations of bonds between protein and lipid (phospholi-
Components from TNBP-treated rabies virus

pid) envelope components, resulting in the release of the latter from the virus particles. Thus, the fate of the virus lipids (phospholipids) may indicate whether and to what extent a certain reagent is able to affect the integrity of the viral envelope.

When $[^{14}C]$-steaeric acid-labelled rabies virus was treated with 0.1% TNBP in the presence of 0.1% Tween 80 for 2 min. at room temperature, only about 24% of the radioactive label was released from the virus particles. This was revealed by rate zonal centrifugation of the treated virus under conditions identical to those indicated for Fig. 2. However, when the virus was treated for 1 hr at 37°, essentially all the label was recovered in the top fraction of the sucrose gradient (Fig. 2). Similar results were obtained with $[^{3}H]$-choline-labelled virus (Fig. 3). These results suggest that TNBP does release the lipids and phospholipids from the virus particles. Intact and treated $[^{3}H]$-choline-labelled virus particles were also submitted to isopycnic centrifugation in CsCl gradients. The intact virus banded at a density of about 1.244 g./cm.³. The radioactive label corresponding to the treated virus was recovered on the top of the gradient and therefore had a density of 1.173 g./cm.³ or less (Fig. 4). Since most of the proteins from the TNBP-treated rabies virus were recovered at a much higher density, the released phospholipids do not appear to remain associated with the virus proteins, at least under the conditions used for isopycnic banding.

Fate of virus RNA during TNBP treatment

$[^{14}C]$-uridine-labelled purified rabies virus, whether or not treated with TNBP, sedimented at approximately the same speed during rate zonal centrifugation in sucrose gradients (Fig. 5). The sedimentation coefficient of the nucleocapsid is about 3 times less than that of the whole virus (Sokol et al. 1969; Neurath et al. 1966). Therefore, the RNA-containing and lipid-deficient particles obtained by TNBP-treatment must contain other proteins in addition
to those of the nucleocapsid. Incubation of the intact or TNBP-treated virus with RNAase A (100 μg./ml., Worthington Biochemical Corporation, Freehold, New Jersey) for 30 min. at 37° failed to alter the distribution of radioactivity across the gradients (Fig. 5). Therefore, the virus RNA in the 'modified' particles is shielded against the enzyme, as was shown before for the viral nucleocapsid itself (Sokol et al. 1969).

![Fig. 2](image-url)  
**Fig. 2.** Distribution of radioactivity in fractions after rate zonal centrifugation of intact (a) and TNBP-treated (b) purified [14C]-stearic acid-labelled rabies virus. Conditions for centrifugation are described under Fig. 1.

![Fig. 3](image-url)  
**Fig. 3.** Distribution of radioactivity in fractions after rate zonal centrifugation of intact (a) and TNBP-treated (b) purified [3H]-choline-labelled rabies virus. Conditions for centrifugation are described under Fig. 1.

**Further elucidation of the effect of TNBP using [14C]-amino acid-labelled and [14C]-d-glucosamine-labelled virus**

The results just presented indicate that TNBP treatment releases the lipid components from rabies virus. To determine to what extent TNBP also affects the virus proteins, treated and untreated [14C]-amino acid-labelled virus was submitted to rate zonal centrifugation. About 40% of the radioactive label from the treated virus was recovered on the top of the gradient (Fig. 6) in six different experiments. The particles corresponding to the other 60% of the radioactivity sedimented at about the same rate as the intact virus and gave a peak at a density of 1.304 g./cm.³ while the intact virus banded at a density of 1.244 g./cm.³ when centrifuged to equilibrium in a CsCl gradient (Fig. 7).

The major structural protein of the rabies virus envelope is a glycoprotein (Sokol et al. 1971). In attempts to demonstrate whether or not treatment with TNBP selectively removes this protein from the virus particles, the effect of this reagent on [14C]-glucosamine-labelled
Components from TNBP-treated rabies virus

virus was studied. Results of 6 experiments revealed that about 82% of the radioactivity was released. The particles containing the residual radioactivity again sedimented in sucrose gradients about equally fast as intact rabies virus (Fig. 8) and had a buoyant density approximately 0.055 g/cm² higher than the intact virus.

Fig. 4. Distribution of radioactivity in fractions after isopycnic centrifugation (35,000 rev./min. for 48 hr at 15°C, rotor SW 65) of intact (a) and TNBP-treated (b) purified [3H]-choline-labelled rabies virus.

Fig. 5. Distribution of radioactivity in fractions after rate zonal centrifugation of purified [14C]-uridine-labelled rabies virus (a) treated with RNAase, (b) treated with TNBP, and (c) treated with TNBP and subsequently with RNAase. The profile of radioactivity corresponding to the intact virus (not treated with RNAase) was identical to that shown in (a). Conditions for centrifugation are described under Fig. 1.

Prolongation of the treatment of [14C]-amino acid-labelled or [14C]-glucosamine-labelled virus up to a total of 16 hr, failed to release significant additional amounts of radioactive material. Tween 80 or TNBP alone did not affect the integrity of the virus particles. Virus suspended in tissue culture medium was as sensitive as purified virus to the combined effect of Tween 80 and TNBP.

Radioactively labelled material obtained from the top of the sucrose gradients (see Fig. 6 and 8) (hereafter referred to as ‘top component’) was recovered in the void volume fractions when submitted to gel filtration on Sephadex G-100. This top component displayed a considerable heterogeneity during rate zonal centrifugation in sucrose gradients under various conditions, making it impossible to determine even its approximate sedimentation coefficient.
Polypeptide composition of intact rabies virus and of products resulting from its treatment by TNBP: RNA-containing particles and 'top component'

To confirm that definite protein species are removed from the virus and to identify these particular proteins, the polypeptide composition of the intact virus, of particles containing RNA, and of the top component was investigated.

![Fig. 6](image1.png)  ![Fig. 7](image2.png)

Fig. 6. Distribution of radioactivity in fractions after rate zonal centrifugation of intact (a) and TNBP-treated (b) purified [14C]-amino acid-labelled rabies virus. Conditions for centrifugation are described under Fig. 1.

Fig. 7. Distribution of radioactivity in fractions after isopycnic centrifugation (35,000 rev./min. for 48 hr at 15°, rotor SW 65) of intact (a) and TNBP-treated (b) purified [14C]-amino acid-labelled rabies virus. The TNBP-treated material corresponds to the faster sedimenting peak in Fig. 6b.

Electrophoresis of intact WI-38 cell-grown rabies virus in polyacrylamide gels in the presence of SLS and ME (Fig. 9a) revealed at least six distinct polypeptides (tentative molecular weights are given in parentheses): NP1 (58,000) and NP2 (47,000), a major and a minor component of the nucleocapsid (compare with Fig. 9b); GP1 (78,000) and GP2 (65,000), two glycoproteins differing markedly in the extent of glycosylation (as is evident from the distinct ratio of radioactivity due to glucosamine and amino acids, respectively); and MP (35,000) and CP (22,000). (The choice of these symbols will be explained in the Discussion.) GP2 itself is probably heterogeneous with respect to the extent of glycosylation, since the peaks corresponding to radioactive glucosamine and amino acids were shifted in repeated experiments. The polypeptides identified in the particles containing RNA were NP1, NP2, MP and CP (Fig. 9c). GP1 and GP2 were also present in residual amounts. The top component consisted of GP1, GP2 and apparently of some MP (Fig. 9d), which repeatedly appeared as a broader peak here than in (Fig. 9a, c).
Fig. 8. Distribution of radioactivity in fractions after rate zonal centrifugation of intact (a) and TNBP-treated (b) purified [14C]-glucosamine-labelled rabies virus. Conditions for centrifugation are described under Fig. 1.

Fig. 9. Separation of rabies virus polypeptides by electrophoresis in polyacrylamide gel. (a) Intact virus; (b) nucleocapsid; (c) RNA-containing particles derived from rabies virus by treatment with TNBP; (d) top component. ——, Radioactivity corresponding to [14C]-amino acid label; ○—○, radioactivity corresponding to [3H]-glucosamine label. Radioactive material preceding peak GP 1 (a) probably represents aggregates of polypeptides. Low levels of radioactivity (about twice the background level) due to the [3H]-glucosamine label were detected in the polypeptide pattern of the RNA-containing particles (c) at positions corresponding to GP 1 and GP 2.
The incomplete release of polypeptides GP1 and GP2 and of some MP from the virus suggest that the particles containing RNA might not be all exactly alike. Electron-microscopical observation confirmed this.

Electron microscopy of the particles containing RNA

To correlate the TNBP-induced biochemical alterations with changes in the morphologic features of rabies virus, the purified RNA-containing particles were submitted to electron microscopy and compared with intact virus. The intact and TNBP-treated particles were similar in size and general shape. However, the treated particles had been penetrated by the

Fig. 10. (a) Purified intact rabies virus. All particles were endowed with surface projections. (b) and (c) Rabies virus treated with 0.1% TNBP and 0.1% Tween 80 at 37°C for 1 hr. The particles have been penetrated by the negative stain. Most of the surface projections are missing. Other envelope components seem to have been leached out from some parts of the virus surface (arrows). An occasional skeleton-like structure with a clearly outlined helix can be seen (c). All specimens were negatively stained with 0.5% ammonium molybdate.
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stain, and lacked most of the surface projections (Fig. 10). The residual projections were
most apparent at the hemispherical ends of the particles. The particles were heterogeneous
with respect to the quantity of residual spikes. Other virus components seem to have been
leached out at some areas on the surface. An occasional skeleton-like structure with a clearly
outlinked helix was seen.

Characterization of bromelain-treated virus.

Treatment of several enveloped viruses with suitable proteolytic enzymes has been shown
to release both the glycoprotein(s) and the surface projections, thus giving evidence for their
identity (Compans et al. 1970; Compans, 1971; Schulze, 1970; Chen, Compans & Choppin,
1971). In order to strengthen the evidence that such identity applies to rabies virus, the

Fig. 11. Separation of polypeptides from bromelain-treated [14C]-amino acid-labelled rabies virus
by polyacrylamide gel electrophoresis. Arrows indicated the positions of peaks corresponding to
the polypeptide species identified in intact rabies virus. No peaks of radioactivity were observed with
bromelain-treated [3H]-glucosamine-labelled virus.

properties of bromelain-treated virus were investigated. The particles retained 41%, 74%
and 75%, respectively, of the radioactivity of the intact amino acid-labelled, choline-labelled
and stearic acid-labelled virus. The apparent partial loss of lipids can probably be attributed
to an increased lability of the treated particles, resulting in the disruption of some of them.
Assuming that this interpretation is correct, the treated particles contained about 55% of
the protein present in the intact virus. Analysis of the polypeptide composition of these
particles revealed that the glycoproteins GP1 and GP2 (representing 43% of the radioactivity
of intact [14C]-amino acid-labelled virus) were virtually absent, while all the other
structural proteins of rabies virus were retained (Fig. 11). The treated particles, some of which
had a distorted shape or were showing signs of starting disruption, lacked the surface
projections (Fig. 12). In polypeptide composition these spikeless particles resembled the
particles containing RNA obtained by treatment of rabies virus with TNBP, but they
sedimented in sucrose gradients about 1.6 times slower (Fig. 13) and had a buoyant density
of approximately 1.22 g./cm.³ (Fig. 14). They were non-infectious and failed to induce virus-neutralizing antibodies in immunized mice, while an equivalent amount of untreated control virus after a 200-fold dilution (= 50% titration end-point) still protected 50% of mice against challenge with the live virus.

Fig. 12. Spikeless particles obtained by treatment of rabies virus with bromelain. Negatively stained with 0.5% ammonium molybdate.

DISCUSSION

The present paper describes some of the results of our efforts to develop a subviral rabies vaccine suitable for application in humans. For this reason, WI-38 human diploid cells were chosen to propagate the virus. To disrupt the viral envelope, we chose TNBP because of our good experience with this organic solvent in preparing influenza vaccines having reduced pyrogenicity as compared with vaccines consisting of intact viruses (Neurath & Rubin, 1971). TNBP released the influenza envelope components in the form of more immunogenic aggregates rather than as less immunogenic monomers. Hence, it is not surprising that similar
treatment of rabies virus failed to release all of the envelope proteins from the virus particles. We avoided the use of sodium deoxycholate, which more drastically disintegrates the envelopes of lipid-containing viruses (see Neurath & Rubin, 1971; Cartwright, Smale & Brown, 1970b) and results in products that are less immunogenic than the intact viruses. It was recently shown that the nonionic detergent Nonidet P-40 removes the projections from the viral surface (Crick & Brown, 1970) and releases the glycoprotein from the rabies virus (Gyorgyi & Sokol, 1971). Rabies virus particles treated with diethyl ether in the presence of Tween 80 remain largely intact (Crick & Brown, 1970), although the infectivity of the virus is considerably reduced by such treatment (Turner & Kaplan, 1967).

![Graph](image1)

![Graph](image2)

Fig. 13. Rate zonal centrifugation (21,000 rev./min. for 17 min., Spinco rotor SW 25) of bromelain-treated [14C]-amino acid-labelled rabies virus particles in a 10 to 25% sucrose gradient. Fraction 1 = bottom of gradient. Arrow indicates the position of the peak of radioactivity corresponding to the intact virus or to the TNBP-treated virus centrifuged under identical conditions. The same results were obtained with either [14C]-stearic acid-labelled or [1H]-choline-labelled virus treated with bromelain.

Fig. 14. Isopycnic centrifugation (35,000 rev./min. for 48 hr, Spinco rotor SW 65) of bromelain-treated [14C]-amino acid-labelled rabies virus particles in a CsCl gradient. Solid and dotted arrows indicate the positions of the peaks of radioactivity corresponding to intact and TNBP-treated viruses, respectively, centrifuged under identical conditions. The same results were obtained with either [14C]-stearic acid-labelled or [1H]-choline-labelled virus treated with bromelain. The minor peak of radioactivity at the bottom of the gradient probably corresponds to the viral nucleocapsids released from a small part of the bromelain-treated particles during centrifugation.

About 40% of the total protein was released from rabies virus during treatment by TNBP. The residual particles, containing all the viral RNA, still sedimented in sucrose gradients like intact virus. This can be ascribed to the fact that these particles had the approximate size and shape of the intact virus but had a considerably higher density. The increase in density can be attributed to the release of lipids. The experiments with [14C]-stearic acid-labelled and [1H]-choline-labelled virus also suggested that lipids are extracted from the virus by TNBP. However, since these radiisotopes would not be incorporated into all classes of lipids, it is not completely certain that all lipids are completely removed from the particles containing RNA. On the other hand, the lower density and sedimentation rate of the bromelain-treated particles as compared with intact virus can be ascribed to their lower protein content and increased lipid/protein ratio.
The buoyant density in CsCl of rabies virus grown in WI-38 cells, as determined by iso-pycnic banding of the radioactive virus, was 1.244 g./cm. This figure is considerably higher than that reported before for infectious virus (Neurath et al. 1966). This discrepancy may be attributed to the different cells used for propagation of the virus and/or to the fact that the virus used in the preceding experiments was not purified and probably remained associated with components of the tissue culture medium (Sokol et al. 1968).

The PM strain of rabies virus, grown in human diploid WI-38 cells had a polypeptide composition similar to that of the HEP FLURY and ERA strains, grown in BHK cells (Sokol et al. 1971). However, two glycoproteins – a minor one (GP1), and a major one (GP2), with tentative molecular weights of 78,000 and 65,000, respectively – could be distinguished instead of a single glycoprotein (molecular weight 80,000), observed by Sokol et al. (1971). The dissimilarity might have been caused by the viruses having been grown in different cells containing distinct species (or at least distinct levels) of glycosyl transferases, since it has been suggested that the carbohydrate portion of viral glycoproteins is host-specified (Grimes & Burge, 1971). On the other hand, the glycoproteins of distinct rabies virus strains may be inherently different. This possibility is supported by the recent isolation of a temperature-sensitive mutant of vesicular stomatitis virus displaying defective glycosylation of one structural protein at nonpermissive temperatures (Printz & Wagner, 1971). Further experiments are required to clarify this question. The lower molecular weights of the structural polypeptides in this than in the study of Sokol et al. (1971) might perhaps be ascribed to the distinct cell types in which the viruses were grown, in analogy with observations made with influenza viruses (Etchison et al. 1971; Haslam et al. 1970).

From the estimated molecular weights of the polypeptides and the distribution of the [14C]-amino acid label across the gels, the approximate molecular proportions of these polypeptides can be assigned as follows:

\[ \text{GP1:GP2:NP1:NP2:MP:CP} = 0.06:1:0.7:0.12:0.51:0.96. \]

Thus, the proportionality between the non-nucleocapsid polypeptides GP1 + GP2, MP and CP is about 2:1:2. This agrees with the data shown in Table 2 in the paper by Sokol et al. (1971).

The TNBP treatment resulted predominantly in the release of the glycoproteins GP1 and GP2. Since the treatment also removed most of the projections from the virus surface, the virus spikes may be tentatively identified with the virus glycoproteins. This conclusion is strengthened by the observation that both glycoproteins and the projections are removed from the virus by treatment with bromelain. Furthermore, since the spikeless particles obtained by the bromelain treatment, unlike the 'top component' from the TNBP treatment, do not induce the formation of virus neutralizing antibodies in immunized animals, these viral projections must carry the antigenic sites for the reaction with the virus neutralizing antibodies. In the case of another rhabdovirus, vesicular stomatitis virus, it was also demonstrated that the surface projections of the virus are responsible for producing virus neutralizing antibodies (Cartwright, Smale & Brown, 1969, 1970a). The particles containing RNA obtained by treatment with TNBP also induced virus neutralizing antibodies, and this is undoubtedly due to the presence of the residual surface projections. They also seem to retain about 0.1% of the infectivity of the intact virus, while the bromelain-treated particles were noninfectious. Thus the surface projections seem to be essential for infectivity.

In addition to the glycoproteins, another polypeptide (MP) appears to be partly released from the virus particles by TNBP, while the smallest non-nucleocapsid polypeptide (CP) remains untouched. We thus believe that MP is an envelope protein which combines with
Components from TNBP-treated rabies virus

the viral lipids within the intact virus particles to form lipoprotein micelles. Probably MP (= micellar protein) is bonded to another underlying protein, CP. The latter may be responsible for holding the nucleocapsid in helical configuration within the bullet-shaped skeleton-like particles shown in Fig. 10(c). This protein may therefore be considered a core protein (= CP).

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REFERENCES


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