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Biochemical Studies on the Phlebotomus Fever Group Viruses (Bunyaviridae Family)

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Analyses of the virion polypeptides and genomes of several Phlebotomus fever group viruses, Karimabad, Punta Toro, Chagres, and the sandfly fever Sicilian serotype viruses, have estblished that they are biochemically similar to the accepted members of the Bunyaviridae family. Like snowshoe hare virus (a member of the California serogroup of the *Bunyavirus* genus of the Bunyaviridae family), Karimabad, Punta Toro, Chagres, and the sandfly fever Sicilian serotype viruses all have three viral RNA species, designated large (L), medium (M), and small (S). Oligonucleotide fingerprint analyses of Karimabad and Punta Toro virus RNA species indicated that their L, M, and S RNA species are unique. By polyacrylamide gel electrophoresis it was determined for Karimabad virus that the apparent molecular weights of its L, M, and S RNA species are 2.6×10^6, 2.2×10^6, and 0.8×10^6, respectively. For Punta Toro virus, the apparent molecular weights of its L, M, and S RNA species are 2.8×10^6, 1.8×10^6, and 0.75×10^6, respectively. The major internal nucleocapsid (N) protein of Karimabad virus was found to have a molecular weight of 21×10^3. A similar polypeptide size class was identified in preparations of sandfly fever Sicilian serotype, Chagres, and Punta Toro viruses. The Karimabad virus glycoproteins formed the external surface projections on virus particles and could be removed from virus preparations by protease treatment. The glycoproteins in an unreduced sample could be resolved into two size classes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They had apparent molecular weights of 62×10^3 and 50×10^3 in continuous polyacrylamide gels. When Karimabad virus preparations were reduced with 1% β-mercaptoethanol, prior to resolution by continuous polyacrylamide gel electrophoresis, all the viral glycoprotein was recovered in a single size class, having an apparent molecular weight of 62×10^3. Two or three major virion polypeptides have been identified in preparations of Punta Toro, Chagres, and sandfly fever Sicilian serotype viruses.

The Bunyaviridae family of arthropod-borne viruses has 89 registered members in the genus *Bunyavirus*, formerly known as the "Bunyamwera supergroup of viruses" (1, 29, 30). The type virus of the genus (and the family) is Bunyamwera virus (1, 8, 9, 29, 30). Another 56 viruses, including the Phlebotomus fever (PHL) group viruses and Uukuniemi virus, are considered as possible members of the family (1, 29, 30). Many of these possible members are morphologically and morphogenically similar to the accepted members of the genus (15, 20), so that they have been termed bunyavirus-like viruses.

The 11 serogroups of viruses in the *Bunyavirus* genus have been grouped together on the basis of serological cross-reactivities (1, 29, 30, 36). Certain, but not necessarily all, members of each serogroup are cross-reactive with particular, but not necessarily all, members of another serogroup. This has led to the concept of the Bunyamwera supergroup of viruses (20, 29, 30). The limited molecular and genetic studies of bunyaviruses that have been published have substantiated that the viruses in the supergroup are structurally comparable to one another (7, 10-14, 17, 19, 21, 22, 24-28, 32, 33, 35; L. H. El Said et al. Am. J. Trop. Med. Hyg., in press). The principal criterion which sets these viruses apart from other families of RNA viruses is the fact that the genetic information of bunyaviruses is negative sense and is resident in three segments of RNA (5-7, 10-14, 17, 22, 24, 26-28, 31; El Said et al., in press).

Other than 6 unassigned viruses, the 56 pos-

sible members of the Bunyaviridae family have been placed into 12 serogroups (1, 29, 30). The largest serogroup is the PHL group, which has 22 members (1, 16, 34). Although morphologically the PHL group viruses are comparable to one another (20), and to other members of the family, no biochemical or genetic evidence has been published to substantiate the placement of these viruses in the Bunyaviridae family.

We report in this paper structural analyses of certain PHL group viruses and show that these viruses have a segmented genome resident in three species of RNA. The virion polypeptides of PHL group viruses are comparable to those of Uukuniemi and the Bunyamwera supergroup viruses. These studies therefore support the placement of the PHL group viruses in the Bunyaviridae family.

MATERIALS AND METHODS

Reagents. Radioisotopes were purchased from ICN, Irvine, Calif. α-Chymotrypsin was obtained from Sigma Chemical Co., St. Louis, Mo.

Viruses and cells. Snowshoe hare, Chagres, sandfly fever Sicilian serotype, Punta Toro (PT), and Karimabad (KAR) viruses were obtained as mouse brain-passaged virus from R. Shope, Yale Arbovirus Research Unit, New Haven, Conn. Vero cells were obtained from R. Tesh, Pacific Research Station, Honolulu, Hawaii.

Plaque assays, growth, and purification of viruses. Snowshoe hare virus was grown in BHK-21 cells in the presence of sodium $^{32}P_i$, [^{3}H]leucine, or ^{14}C-labeled amino acids and was purified as described previously (10, 13, 21-23). The PHL group viruses were each plaque purified at 35°C by use of confluent monolayers of Vero cells and an overlay which consisted of medium 199 lacking phenol red (Microbiological Associates, Walkersville, Md.) mixed with 1% (wt/vol) purified agar (Difco), essential vitamins and amino acids (1 × concentration, Microbiological Associates), 1% (vol/vol) dimethyl sulfoxide (Sigma Chemical Co.), 10 U of penicillin/ml, 10 μg of streptomycin/ml, 10% (vol/vol) heat-inactivated fetal calf serum (HEM, Rockville, Md.), and 0.02% DEAE-dextran (Sigma), buffered with 0.3% (wt/vol) sodium bicarbonate. The dimethyl sulfoxide and DEAE dextran are required for obtaining plaques with the sandfly fever viruses, but not for the other PHL group viruses used in this study (J. M. McCown, W. E. Brandt, W. H. Bancroft, and P. K. Russell, Am. J. Trop. Med. Hyg., in press). The plaque assays were overlaid 4 to 7 days later (depending on the virus strain) with 1% (wt/wt) purified agar (Difco) made up in 0.85% (wt/vol) saline containing 0.133 mg of neutral red/ml. Plaques (1 to 4 mm in diameter) were counted and picked 1 day later.

Working stocks of the PHL group viruses were obtained by infecting Vero cells with the mouse brain-passaged virus or the virus eluted from a plaque and growing them for 3 days at 35°C. The growth medium consisted of Eagle basal medium (Earle salts, Microbiological Associates), containing 5% (vol/vol) heat-inactivated fetal calf serum (HEM), 1% (wt/vol) L-glutamine, 100 U of penicillin/ml, and 0.1 mg of streptomycin/ml. After two and three serial passages in Vero cells, working stocks of the PHL viruses were obtained with titers of 2.2×10^7 PFU/ml (Sicilian sandfly fever), 2.1×10^7 PFU/ml (Chagres), 6.5×10^7 PFU/ml (KAR), and 5.3×10^8 PFU/ml (PT). Virus stocks were kept at −70°C in the presence of 20% (vol/vol) heat-inactivated fetal calf serum.

Labeled virus preparations were obtained by growing the PHL group viruses for 3 days at 35°C in growth medium containing [^{3}H]leucine (10 μCi/ml), ^{14}C-labeled amino acid mixture (1 μCi/ml), [^{3}H]uridine (20 μCi/ml), sodium $^{32}P_i$ (200 μCi/ml), or [^{3}H]glucosamine (5 μCi/ml). The viruses were purified by polyethylene glycol-NaCl precipitation followed by two 90-min cycles of glycerol-potassium tartrate gradient centrifugation as described previously (21, 23). After they were harvested from the second gradient, the viruses were dialyzed overnight against either 0.01 M Tris-hydrochloride buffer, pH 7.4, or 0.15 M NaCl, in 0.01 M Tris-hydrochloride buffer, pH 7.4. In initial experiments virus preparations were purified by successive glycerol-potassium tartrate and sucrose gradient centrifugation followed by pelleting (21, 23). Since this latter procedure proved deleterious for obtaining sufficient yields of intact virus preparations, the former procedure was subsequently adopted (see Discussion).

Polyacrylamide gel electrophoresis for resolving viral polypeptides. Virus preparations in 0.01 M Tris buffer were dissociated by 1% (wt/vol) sodium dodecyl sulfate (SDS), 1 M urea, and 0.01 M sodium phosphate, pH 7.0, and were incubated in the presence or absence of 1% (vol/vol) β-mercaptoethanol at 60°C for 30 min. The viral polypeptides were resolved by electrophoresis in continuous 8% (wt/vol) polyacrylamide gels containing 0.1 M sodium phosphate buffer, pH 7.0, 0.1% SDS, and 6 M urea as described previously (23). The distribution of radioactivity was determined after slicing each gel into 1-mm sections and eluting the radioactivity in Protosol (New England Nuclear Corp., Boston, Mass.) for 18 h at 37°C before addition of a toluene-based scintillation cocktail. The resolution of viral polypeptides by discontinuous polyacrylamide slab gel electrophoresis was performed as described elsewhere (13, 21, 23). After electrophoresis, gels were fluorographed by the procedure of Bonner and Laskey (4) by treatment first with dimethyl sulfoxide, then dimethyl sulfoxide containing 2,5-diphenyloxazole, and finally water. The gels were dried and autoradiographed. Autoradiographs were scanned at 550 nm in a Schoeffel double-beam spectrodensitometer (Schoeffel Instrument Corp., Westwood, N.J.).

Polyacrylamide gel electrophoresis for resolving RNA species. Labeled virus or cell preparations were extracted for RNA, and the RNA species were resolved by electrophoresis in 2.4% gels of polyacrylamide as described previously (2, 13, 22).

Resolution of the viral RNA species by SDS-sucrose gradient centrifugation, RNase T_1 digestion of RNA, and separation of the oligonucleotides by two-dimensional gel electrophoresis. The extraction and purification of ^{32}P-labeled bunyavirus L, M, and S RNA species have been described

previously (10). RNA samples, dissolved in 15 μl of 0.02 M Tris-hydrochloride–0.002 M EDTA (pH 7.4), were digested with 10 U of RNase T_1 at 37°C for 30 min, and the resulting oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis (10). After electrophoresis, each gel was autoradiographed to obtain the oligonucleotide fingerprint (10).

Electron microscopy. Samples were applied to 300-mesh copper grids with carbon-coated Formvar films and stained with 2% (wt/vol) sodium phosphotungstate, pH 6.2. Specimens were examined in a Philips 301 electron microscope.

Polyethylene glycol-dextran T-500 phase separation of dissociated virus components. The procedures used to phase-separate viral nucleocapsids from solubilized virion components after treatment with Triton X-100 have been described (3).

RESULTS

Growth capabilities of KAR, PT, Chagres, and Sicilian sandfly fever viruses in Vero cells. When confluent monolayers of Vero cells were infected with KAR virus at multiplicities of infection of 0.1 to 20 PFU per cell, the maximum yields of infectious progeny virus (1×10^7 to 5×10^7 PFU/ml) were obtained 2 to 3 days postinfection for cells incubated at 35 to 38°C. For infections initiated at lower multiplicities of infection (e.g., 0.001 PFU/cell), slightly higher yields of infectious virus were obtained (1×10^8 to 2×10^8 PFU/ml), with maximum titers achieved by 5 days postinfection. Essentially similar results were obtained for Chagres, PT, and Sicilian sandfly fever infections.

Viral RNA species of Sicilian sandfly fever, Chagres, KAR and PT viruses. Central to the question of whether the PHL group viruses are like other members of the Bunyaviridae family is whether they have genomes consisting of three segments of RNA. The presence of a segmented genome for Sicilian sandfly fever, Chagres, KAR, and PT viruses was investigated by analyzing their ^{32}P-labeled viral RNA species by SDS-sucrose gradient centrifugation (Fig. 1). For all four viruses, three peaks of radioactivity were obtained which, in view of their sizes, have been designated large (L), medium (M), and small (S). The sedimentation coefficients of the three RNA species for all four viruses were estimated by comparison to marker BHK-21 28S and 18S rRNA species, run in a parallel gradient, giving values of 29 ± 2S, 24 ± 2S, and 18 ± 2S, for L, M, and S, respectively.

To further characterize the viral RNA species

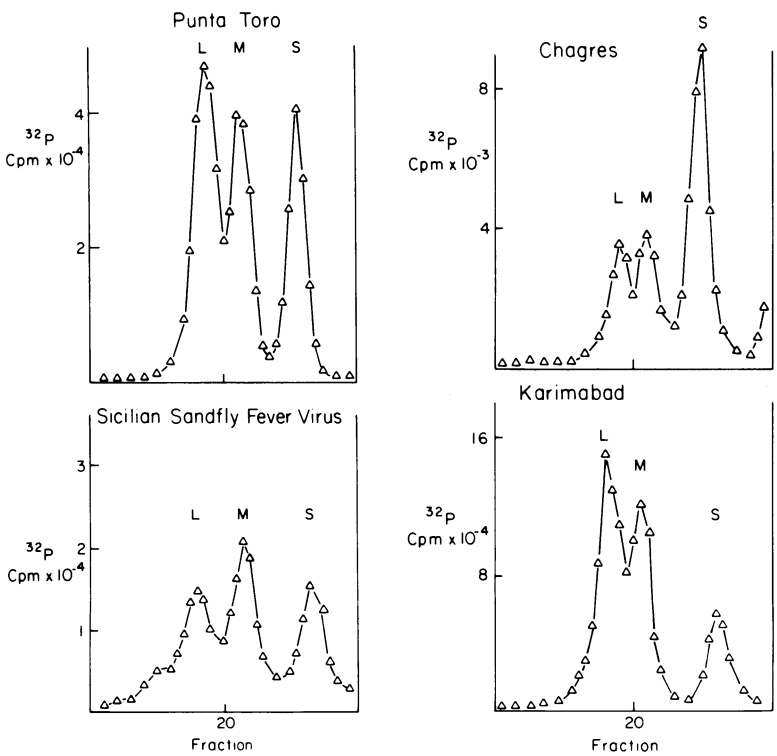

FIG. 1. *Sucrose gradient resolution of the viral RNA species of Punta Toro, Sicilian sandfly fever, Chagres, and Karimabad viruses. The ^{32}P-labeled viral RNA species were resolved by SDS-sucrose gradient centrifugation as described previously (10).*

of KAR and PT viruses, we obtained [^{3}H]uridine-labeled virus preparations and extracted them for RNA. The RNA extracts were subjected to coelectrophoresis in 2.4% polyacrylamide gels with either ^{32}P-labeled BHK-21 28S and 18S rRNA species (Fig. 2) or ^{32}P-labeled snowshoe hare virus RNA (Fig. 3). Three viral RNA species (L, M, and S) were resolved for

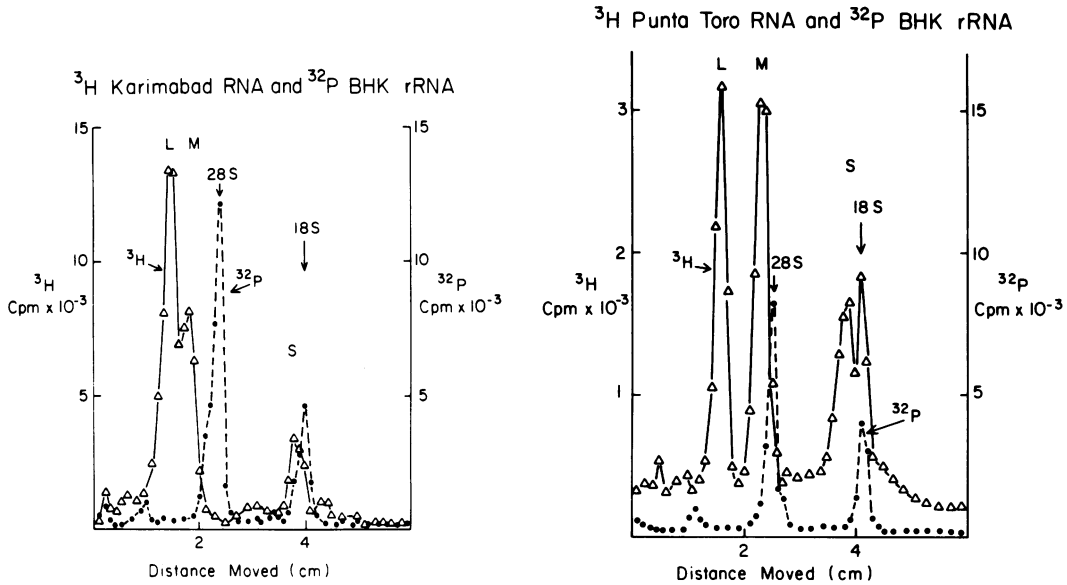

FIG. 2. *Resolution of the viral RNA species of Karimabad and Punta Toro viral RNA species by polyacrylamide gel electrophoresis with marker BHK-21 cell 28S and 18S rRNA species. Samples of [^{3}H]-uridine-labeled viral RNA species with ^{32}P-labeled cell rRNA species were resolved by 2.4% polyacrylamide gel electrophoresis as described previously (2, 22).*

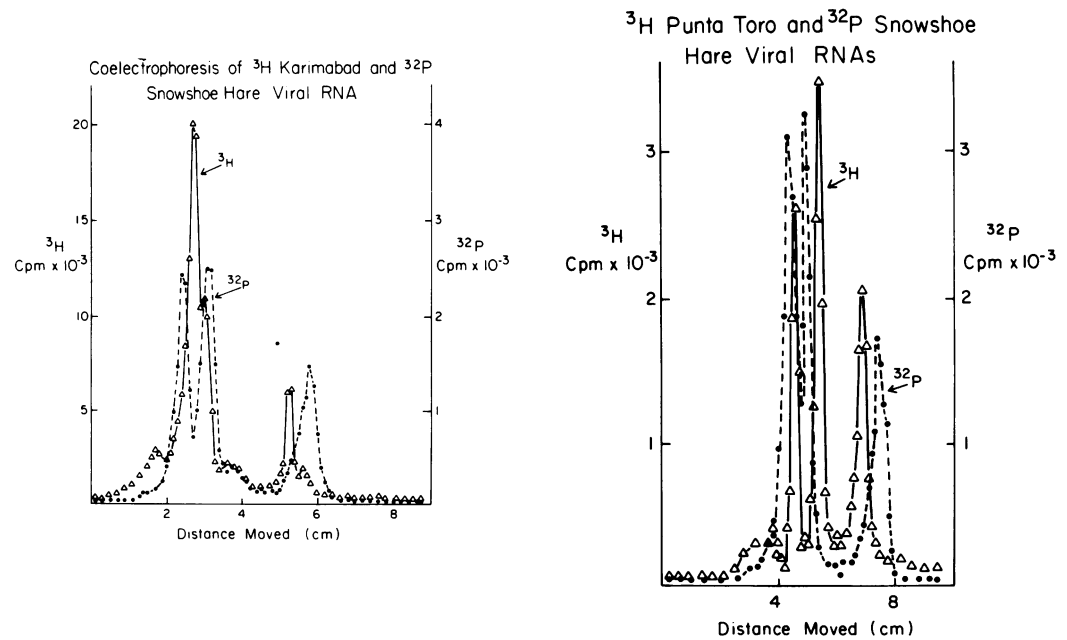

FIG. 3. *Resolution of the viral RNA species of Karimabad and snowshoe hare viruses and of Punta Toro and snowshoe hare viruses by polyacrylamide gel electrophoresis. Samples of [^{3}H]uridine-labeled Phlebotomus fever group viral RNA species and ^{32}P-labeled snowshoe hare virus RNA were resolved by 2.4% polyacrylamide gel electrophoresis as described previously (2, 22).*

KAR virus. The larger two species (L and M) migrated slower than the marker 28S rRNA and were poorly resolved from each other. The KAR S RNA had an electrophoretic mobility slightly slower than that of the 18S RNA marker.

In contrast to the results obtained for KAR virus, the L and M RNA species of PT virus were well separated from each other. Although in the coelectrophoresis with the rRNA species four PT RNA peaks were resolved (Fig. 2), the RNA species which migrated with the marker 18S rRNA was not always found in PT virus RNA extracts (see Fig. 3). It is possible that this 18S species represents contaminating 18S cellular ribosomal RNA.

Estimates of the apparent molecular weights of the KAR and PT virus RNA species have been made from their electrophoretic mobilities (2, 18) relative to the molecular weight and mobilities of the BHK-21 28S and 18S rRNA (1.75×10^6 and 0.7×10^6, respectively) and snowshoe hare virus RNA species (L, 3.0×10^6; M, 1.9×10^6; S, 0.45×10^6). The RNA species of KAR virus were thereby estimated to have molecular weights of 2.6×10^6 (L), 2.2×10^6 (M), and 0.8×10^6 (S). The three RNA species of PT virus were estimated to have molecular weights of 2.8×10^6 (L), 1.8×10^6 (M), and 0.75×10^6 (S).

Oligonucleotide fingerprint analyses of the L, M, and S RNA species of KAR and PT viruses. The three ^{32}P-labeled RNA species of KAR and PT viruses were resolved by SDS-sucrose gradient centrifugation, and the fractions representing the top and leading sides of the L peaks, top and trailing sides of the M peaks, and whole S RNA peaks were recovered and digested with RNase T_1. Each digest was resolved by two-dimensional polyacrylamide gel electrophoresis and autoradiographed (Fig. 4). In the fingerprints presented, the first-dimension electrophoresis was from left to right, and the second dimension from bottom to top. On each fingerprint is indicated the final position of two

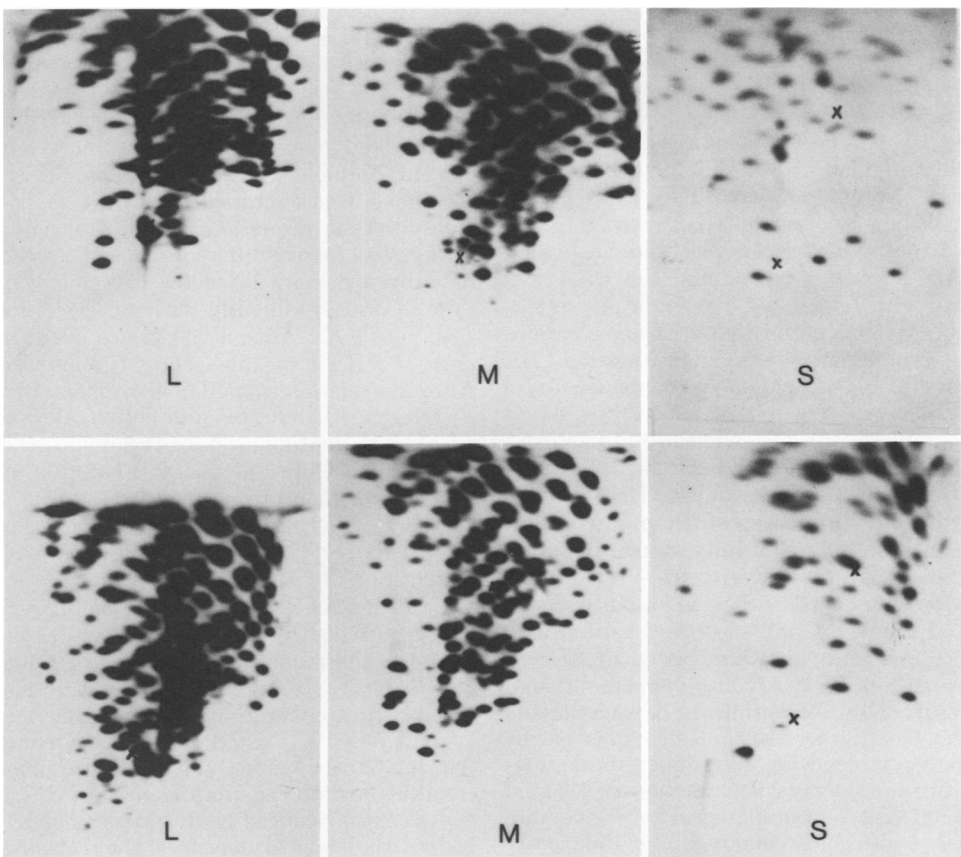

FIG. 4. *Oligonucleotide fingerprint analyses of the L, M, and S RNA species of Karimabad virus (lower three panels) and Punta Toro virus (upper three panels). The oligonucleotide fingerprints of ^{32}P-labeled viral RNA species were obtained as described in the text and elsewhere (10).*

dye markers, bromophenol blue (upper center), and xylene cyanol FF (lower left).

The S RNA fingerprints of either virus were evidently less complex than their respective L or M RNA patterns. The fingerprints of the S, M, or L RNA species of KAR virus did not resemble the corresponding patterns of PT virus, indicating that the viruses have different S, M, and L RNA nucleotide sequences. In the S RNA patterns of both viruses, particular oligonucleotides were evident (e.g., bottom left for KAR S RNA and extreme right center for PT S RNA) which had no major spot counterparts in the respective M or L patterns. Although no S and M or S and L coelectrophoreses were performed, these results indicate that the S RNA species of either virus has a unique nucleotide sequence.

The L and M RNA fingerprints of KAR or PT virus have comparable numbers of large oligonucleotides (e.g., those below the bromophenol blue marker). The M RNA oligonucleotide pattern of either virus is clearly distinct from that of its respective L RNA species (and vice versa). The KAR virus L RNA pattern has both faint spots corresponding in position to the largest major spots in its M (and possibly S) RNA patterns and also has major spots which are not evident as major spots in the M (or S) RNA patterns. The minor spots in the KAR L pattern probably originate from KAR M (and S) RNA species which contaminated the L RNA preparation. When the fractions between the peak KAR L and M RNA species were recovered from the sucrose gradient and the RNA was digested with RNase T_1, a fingerprint was obtained containing both L and M oligonucleotides having approximately equal densities (data not presented). No other major oligonucleotides were evident.

In summary, the oligonucleotide fingerprint analyses indicate that both KAR and PT viruses have three unique species of viral RNA.

Preliminary analyses of the major virion polypeptides of Sicilian sandfly fever, Chagres, KAR, and PT viruses. Preparations of [^{3}H]leucine-labeled Sicilian sandfly fever, Chagres, KAR, PT and snowshoe hare viruses (see Fig. 5 legend) were dissociated by SDS in the presence of 1% (vol/vol) β-mercaptoethanol and resolved by discontinuous polyacrylamide slab gel electrophoresis. The positions of the viral polypeptides were determined by fluorography (4), and the autoradiograms were scanned at 550 nm (Fig. 5). Compared to snowshoe hare virus, each virus had a major band of radioactivity similar in size to the snowshoe hare virus N polypeptide (molecular weight, 21×10^3). Other major and several minor bands of labeled polypeptides were also evident. The approximate molecular weights of these major bands have been estimated by reference to the snowshoe hare species (Sicilian sandfly fever, 57×10^3 and 22×10^3; Chagres, 85×10^3, 54×10^3, and 22×10^3; PT, 65×10^3, 50×10^3, and 23×10^3; KAR, 58×10^3 and 20×10^3).

A sample of SDS-dissociated and β-mercaptoethanol-reduced, [^{3}H]leucine-labeled KAR viral polypeptides was mixed with ^{14}C-amino acid-labeled snowshoe hare virus and resolved by continuous polyacrylamide gel electrophoresis at pH 7.0. Two major viral polypeptide species were identified having apparent molecular weights of 62×10^3 and 21×10^3 (Fig. 6). Another 100×10^3 polypeptide was also observed as well as additional larger-molecular-weight species. Since these other species were not always found in preparations of KAR virus (see Fig. 7), it is possible that they represent contaminating nonviral polypeptides or aggregates of incompletely dissociated viral polypeptides.

Identification of the viral glycoproteins of KAR and Chagres viruses. For snowshoe hare La Crosse, Bunyamwera, and other Bunyamwera and California encephalitis serogroup viruses, two virion glycoproteins, G1 (molecular weight, 110×10^3) and G2 (molecular weight, 38×10^3), and one internal major nucleocapsid protein, N (molecular weight, 19×10^3 to 24×10^3), have been identified (11–14, 19, 21, 25, 35; El Said et al., in press). Similar viral polypeptides (albeit having different molecular weights) have been demonstrated for Uukuniemi virus (30). In order to identify the viral glycoprotein species of KAR virus, it was grown in the presence of [^{3}H]glucosamine and ^{14}C-amino acids. After dissociation with SDS and reduction with β-mercaptoethanol, the viral polypeptides were resolved by continuous polyacrylamide gel electrophoresis. Only the 62×10^3-dalton polypeptide was found to be glycosylated (Fig. 7A).

Effect of reduction by β-mercaptoethanol on the KAR virus glycoproteins. When a preparation of [^{3}H]glucosamine- and ^{14}C-amino acid-labeled KAR virus was dissociated by SDS in the absence of β-mercaptoethanol and resolved by continuous polyacrylamide gel electrophoresis, two glycoprotein species were identified having apparent molecular weights of 62×10^3 and 50×10^3 (Fig. 7B). Since the amounts of the dual-labeled virus and the electrophoretic conditions employed were identical for the reduced and unreduced KAR virus samples (Fig. 7A and B), it was evident from the recoveries of ^{14}C and ^{3}H that all of the radioactivity found in the two glycoprotein bands of the unreduced KAR preparation was present as a single band

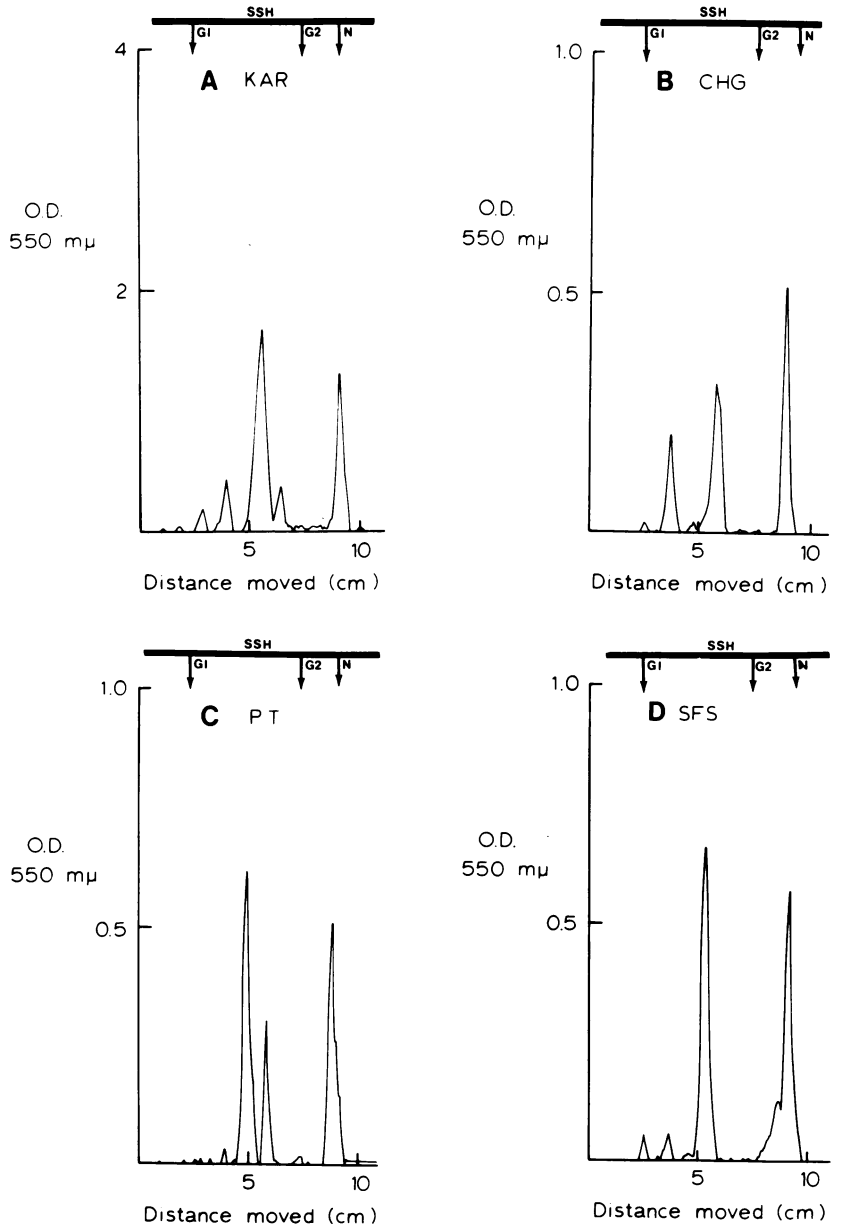

FIG. 5. *Polyacrylamide gel electrophoresis of the virion polypeptides of (A) Karimabad, (B) Chagres, (C) Punta Toro, and (D) Sicilian sandfly fever viruses. Preparations of [^{3}H]leucine-labeled Phlebotomus fever group and snowshoe hare viruses were dissociated with SDS in the presence of 1% β-mercaptoethanol and were resolved by discontinuous slab polyacrylamide gel electrophoresis (13, 21, 23). After fluorography (4), the samples were scanned at 550 nm with a Schoeffel spectrodensitometer. The comparable positions of the snowshoe hare G1, G2, and N viral polypeptides (13) are indicated in the scans of the Phlebotomus fever group virion polypeptides. For these Phlebotomus fever group virus preparations, successive glycerol tartrate and sucrose gradient centrifugation was used followed by virus pelleting. As indicated in the Discussion and Materials and Methods, this procedure was later discontinued as a result of problems encountered in obtaining sufficient yields of intact virus.*

in the reduced viral sample. Two KAR glycoproteins have been observed for unreduced viral polypeptide preparations resolved by electrophoresis in continuous, discontinuous, tube, or slab polyacrylamide gels, or in discontinuous 5 to 20% gradient slab polyacrylamide gels (data

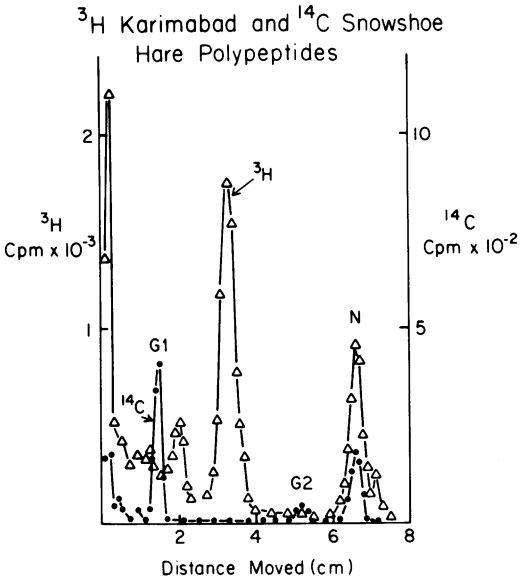

FIG. 6. *Coelectrophoresis of [^{3}H]leucine-labeled Karimabad virus and ^{14}C-amino acid-labeled snowshoe hare virus on continuous polyacrylamide gels. Samples of the two viruses were purified as described in Fig. 5, dissociated by SDS in the presence of 1% β-mercaptoethanol, and resolved by polyacrylamide gel electrophoresis (13, 21, 23). The identities of the three major snowshoe hare virus polypeptides, G1, G2, and N (13), are indicated.*

not shown). Treatment of 10- to 50-μg amounts of SDS-dissociated KAR virus preparations with different concentrations of β-mercaptoethanol (0.01 to 10%, vol/vol) has resulted in the transition from a double to a single glycoprotein size class at concentrations of the reducing agent in excess of 1%.

The effect of omitting a reducing agent from the dissociation mixtures of Sicilian sandfly fever, Chagres, or PT virus has not yet been investigated because of difficulties encountered in obtaining adequate quantities of dual-labeled virus for such experiments.

Location of the viral polypeptides. Electron micrographs of negatively stained KAR virus preparations indicated that virus particles are mostly spherical, 100 ± 10 nm in diameter, including a 10-nm external fringe of surface projections apparently attached to an electron-lucent envelope (Fig. 8A). Occasional deformed particles were also observed. Where phosphotungstate penetrated the particles, the viral envelope could be discerned, but internal structures were not resolved (Fig. 8A). Frequently, virus preparations were found to contain large numbers of aggregated virus particles (Fig. 8B).

The surface projections could be removed from virus particles by proteolytic enzyme treatment (Pronase, 1 mg/ml, 30 min at 30°C; or α-chymotrypsin, 0.125 mg/ml, 30 min at 30°C), giving spikeless particles having diameters of 85 ± 10 nm (Fig. 8C). Because repurification of the spikeless particles by sucrose or glycerol-potassium tartrate gradient centrifugation resulted in considerable losses of the spikeless particles, analyses of their composition were made directly after proteolytic enzyme treatment. Samples of 100-μl volumes of [^{3}H]glucosamine- and ^{14}C-amino acid-labeled KAR virus were treated with 12.5 or 30 μg of α-chymotrypsin at 30°C for 30 min, mixed with 100 μg of bovine serum albumin and SDS (1%, final concentration), and then heated to 100°C for 2 min and immediately resolved by continuous 8% polyacrylamide gel electrophoresis. As shown in Fig. 9, treatment of KAR virus preparations with either concentration of α-chymotrypsin resulted in the digestion of both of the viral glycoprotein size classes. A variety of degradation products were observed in the enzyme-treated samples (Fig. 9).

The nonglycosylated viral polypeptide was unaffected by the proteolytic enzyme treatment. When a similar experiment was performed with Triton X-100 (1%, final concentration)-disrupted virus preparations, the nonglycosylated polypeptide was digested by the enzyme treatments (data not shown).

The fact that chymotrypsin removed the surface projections from virus particles and specifically degraded the viral glycoproteins, but not the nonglycosylated species, indicates that the glycoproteins form the surface projections seen in electron micrographs of virus preparations, and that the 21 × 10^3-dalton, nonglycosylated polypeptide is an internal component.

Identification of the major nucleocapsid protein of KAR virus. The major nucleocapsid protein of La Crosse virus (and other bunyaviruses) is the 19 × 10^3- to 24 × 10^3-dalton polypeptide. In order to determine whether the major nonglycosylated protein of KAR virus is associated with the viral nucleocapsids, a [^{3}H]glucosamine- and ^{14}C-amino acid-labeled KAR virus preparation was dissociated with Triton X-100 (1%, final concentration) in the presence of 2% (wt/vol) ammonium bicarbonate and was mixed with polyethylene glycol and dextran T-500 to phase-separate the viral nucleocapsids from the solubilized virus components (3). The material in the polyethylene glycol and dextran T-500 phases was recovered, dissolved in 1% SDS containing 1% β-mercaptoethanol, 1 M urea, and 0.01 M sodium phosphate buffer, pH 7.0, and resolved by electrophoresis in an 8% continuous polyacrylamide gel. The dextran T-500 phase was found to contain the major non-

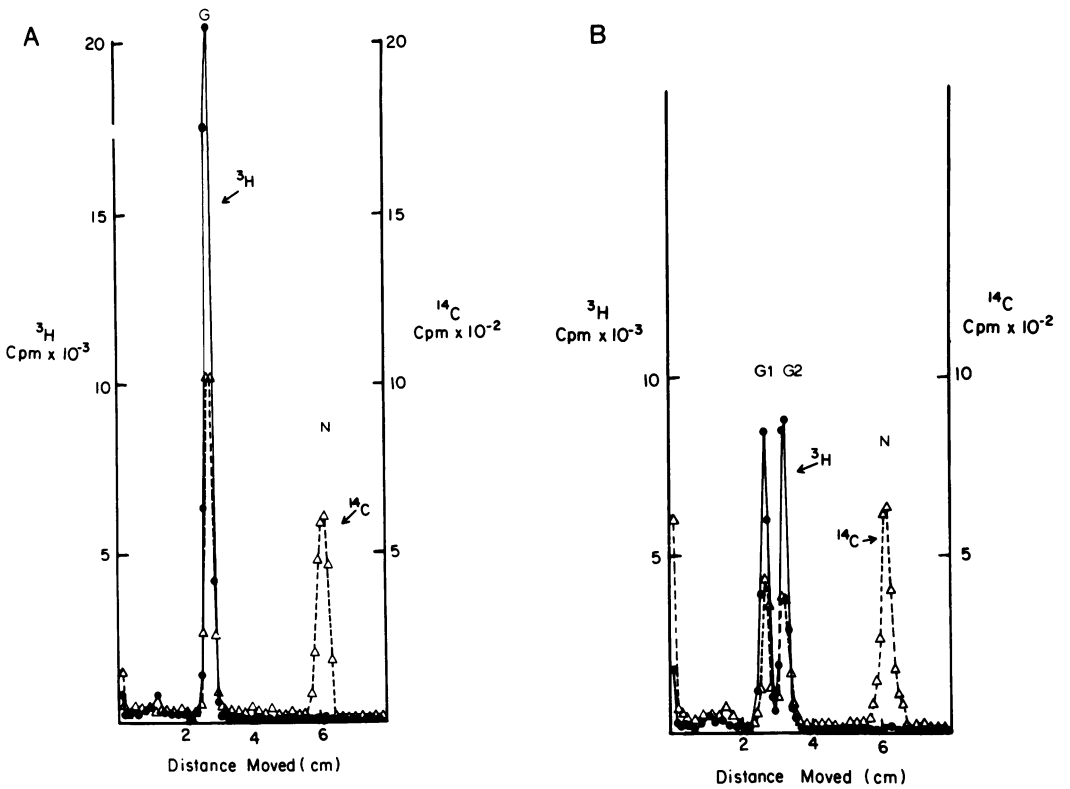

FIG. 7. *Effect of β-mercaptoethanol on Karimabad virus polypeptides and identification of Karimabad virus glycoproteins. Preparations of [^{3}H]glucosamine and ^{14}C-amino acid-labeled Karimabad virus, purified by two successive 90-min cycles of glycerol-potassium tartrate centrifugation, followed by dialysis (see Materials and Methods), was dissociated by SDS in the presence (A) or absence (B) of β-mercaptoethanol. After electrophoresis, the positions and identity of the viral polypeptides were determined.*

glycosylated polypeptide, while the polyethylene glycol phase contained the viral glycoprotein (data not shown).

DISCUSSION

Evidence has been obtained which indicated that, of the PHL group viruses, the KAR, Sicilian sandfly fever, Chagres, and PT viruses structurally resemble accepted members of the Bunyaviridae family. The fact that Bunyamwera supergroup viruses such as La Crosse, Lumbo, and snowshoe hare viruses have tripartite genomes has been established by both sucrose gradient gel electrophoresis and oligonucleotide fingerprinting methods (7, 10, 13, 14, 17, 22; El Said et al., in press). Similar results have been obtained for Uukuniemi virus (26). The results reported in this study, obtained by using the same analytical procedures, support the conclusion that the PHL group viruses also have tripartite genomes.

Although by gel electrophoresis the apparent molecular weights of the S RNA species of KAR and PT viruses (0.75×10^6 to 0.8×10^6) are significantly larger than those of La Crosse or snowshoe hare virus (0.5×10^6 to 0.45×10^6), the complexities of their S RNA fingerprints are similar to those of the S RNA species of La Crosse and snowshoe hare viruses (10). This could be explained if the KAR and PT S RNA species have a high content of guanylic residues, resulting in more RNase T_1 digestion products. Alternatively, it is possible that the electrophoretic behavior of the KAR and PT S RNA species is aberrant as a result of conformational or net charge density factors.

It has been shown that the S RNA species of La Crosse and snowshoe hare viruses code for their respective nucleocapsid proteins (12). Whether the S RNA species of these viruses code for other virus-induced proteins is not known. The KAR and PT viruses both have a nonglycosylated 20×10^3 to 22×10^3-dalton polypeptide which, at least for KAR virus, appears to be nucleocapsid associated. If the KAR S RNA codes only for this protein, then the disparity between its nucleocapsid protein size and the apparent coding capacity of the KAR S

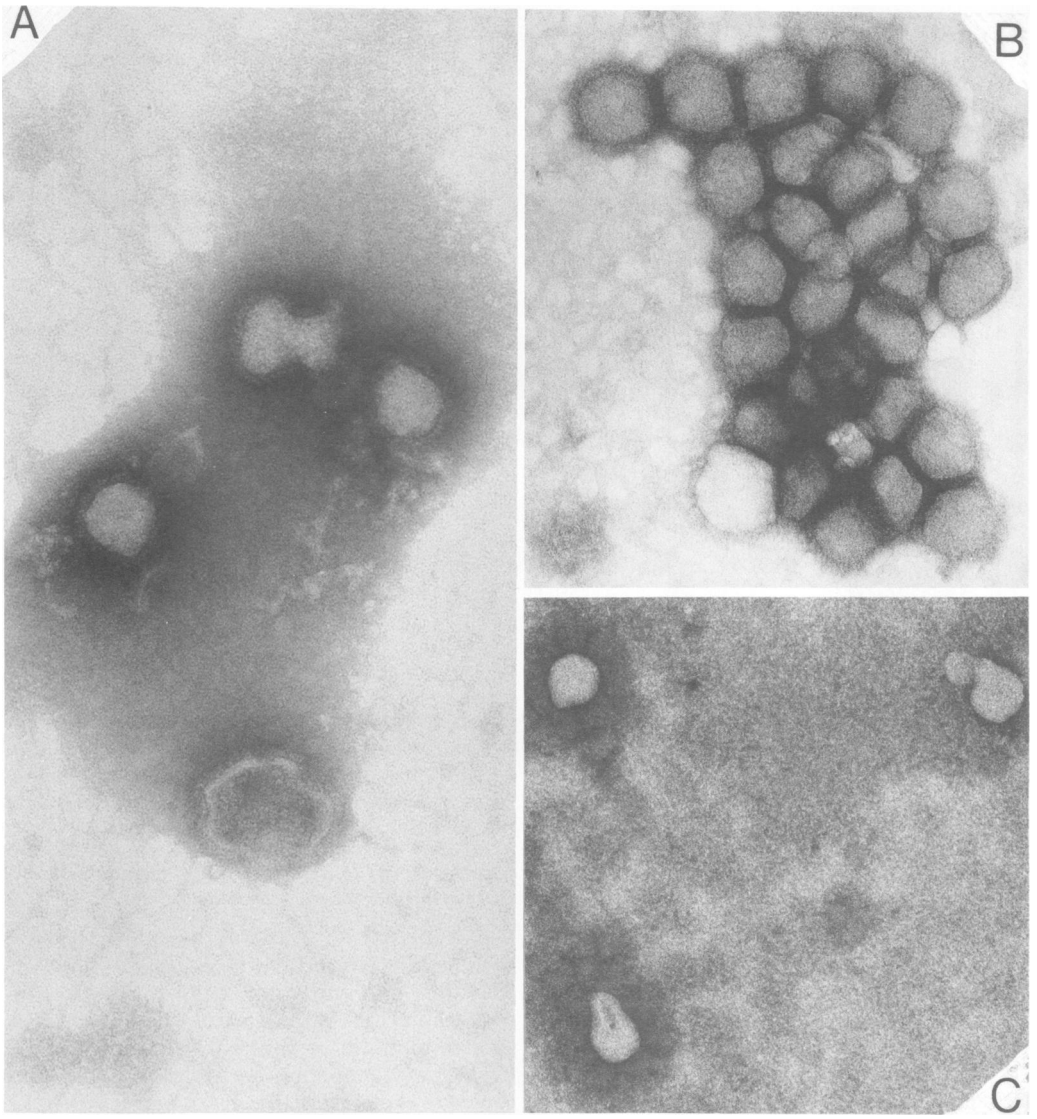

FIG. 8. *Electron micrographs on (A, B) Karimabad virus preparations and (C) spikeless particles. × 75,000. Preparations of Karimabad virus purified as described in Fig. 7 were processed for electron microscopy as described in Materials and Methods. The spikeless virus particles were obtained by α-chymotrypsin treatment (0.125 mg/ml for 30 min at 30°C).*

RNA is even greater than for La Crosse or snowshoe hare virus. If this issue is to be resolved, alternative procedures to determine the KAR S RNA molecular weight will have to be investigated. In addition, it will be necessary to determine whether more than one viral polypeptide is coded for by the S RNA species of the PHL group viruses.

The major viral polypeptides of KAR virus include an internal, nonglycosylated, nucleocapsid-associated, 20 × 10^3-dalton polypeptide and external glycoprotein species which, in nonreduced preparations, resolve into two polypeptide size classes, whereas in reduced samples (i.e., those treated with concentrations of β-mercaptoethanol in excess of 1%) they are recovered as a single size class. The effect of omitting a reducing agent from the dissociation mixtures of other PHL virus preparations upon the electrophoretic behavior of their polypeptides has not been investigated because of difficulties encountered in obtaining sufficient quantities of dual-labeled virus. Preliminary analyses indicate, however, that sandfly fever Sicilian serotype,

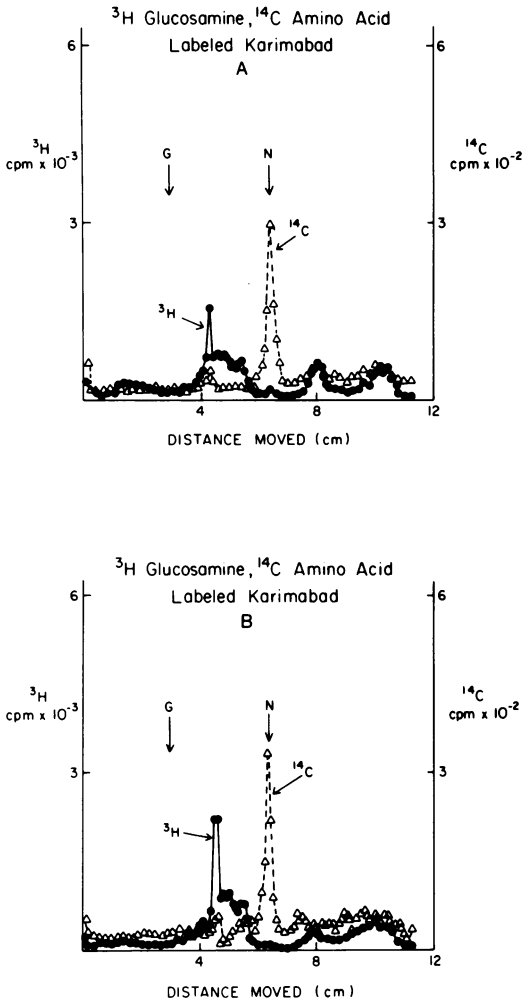

FIG. 9. *Effect of α-chymotrypsin on the viral polypeptides of Karimabad virus. Preparations of [^{3}H]-glucosamine and ^{14}C-amino acid-labeled Karimabad virus (Fig. 7) were treated with α-chymotrypsin at (A) 0.125 mg/ml versus (B) 0.300 mg/ml (final concentration) at 30°C for 30 min, mixed with 0.1 mg of bovine serum albumin and SDS, and heated at 100°C for 2 min. The products were resolved by continuous 8% polyacrylamide gel electrophoresis. A gel subjected to electrophoresis in parallel and containing untreated Karimabad virus polypeptides gave the expected positions of the Karimabad virus G and N polypeptides as indicated in A and B.*

PT, and Chagres viruses all have a major 20 × 10^3- to 24 × 10^3-dalton viral polypeptide as well as one or two major virion polypeptides in the 45 × 10^3- to 92 × 10^3-dalton size range.

A contributing factor to the difficulties in pursuing the polypeptide studies with sandfly fever Sicilian serotype, PT, and Chagres viruses has been the problem encountered in maintaining the structural integrity of their virus particles. This has also been a problem in obtaining KAR virus preparations. We have found that sucrose gradient centrifugation, or extended periods of glycerol-potassium tartrate gradient centrifugation, or high-speed pelleting of KAR virus preparations usually leads to substantial losses of virus particles. Repurification of spikeless KAR virus particles has also proved difficult. The protocol described in Materials and Methods for obtaining KAR virus has consistently led to better yields of virus and more intact virions (see Fig. 7 and 8) than protocols involving successive cycles of glycerol-potassium tartrate and sucrose gradient centrifugation followed by high-speed pelleting, which were, however, used for earlier preparations of KAR virus (see Fig. 5 and 6).

The question of whether KAR virus has one or two glycoproteins remains an unresolved issue. Peptide analyses will be needed to determine the uniqueness of the two glycoprotein species resolved for the unreduced KAR virus preparations. Some differences in the ^{14}C to ^{3}H ratios of the two KAR glycoproteins have been observed from [^{3}H]glucosamine- and ^{14}C-amino acid-labeled KAR virus preparations (see Fig. 7B).

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