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Properties of an Antigenic Glycoprotein Isolated from Influenza Virus Hemagglutinin

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A purified antigen, HABA protein, has been derived from influenza virus concentrates by extraction with denaturing solvents. The protein lacks hemagglutinating activity but binds completely strain-specific, hemagglutination-inhibiting antibodies and induces neutralizing antibodies in experimental animals. Physicochemical characterization of HABA protein identifies it as a single homogeneous glycoprotein with a molecular weight of 78,000. On dissociation with guanidine or sodium dodecyl sulfate, in the presence of reducing agents, only one size of polypeptide with a molecular weight of the order of 40,000 is characteristic of the preparations. The data indicate that HABA protein is a dimer of HA_1 polypeptide of the influenza virus hemagglutinin substructure, and that only trace amounts of other polypeptides are present.

Influenza virus exhibits a complex structure characterized by possession of a lipid protein membrane with spikelike projections extending from the surface. Some seven viral polypeptides have been identified by acrylamide gel electrophoresis, four of which are glycopeptides (4).

A major step in establishing the structure of influenza virus would be identification of those polypeptides associated with its several functional subunits. With respect to the substructure involved in hemagglutination, two polypeptides, HA_1 and HA_2, have been nominated (13). In studies on viral structure, we have isolated a homogeneous protein related to hemagglutinin (7). This protein does not agglutinate erythrocytes but does bind hemagglutination-inhibiting (HI) antibodies. On this basis it is referred to as hemagglutinin-binding antigen or HABA protein. Evidence has been obtained for the presence of only a single size of polypeptide subunit in HABA protein (9).

The present report aims at characterizing the HABA molecule and its constituent polypeptide with reference to composition, molecular dimensions, and immunological properties. The data support the tentative designation of HABA protein as a dimer of HA_1 polypeptide arranged in a conformation retaining immunological specificity.

MATERIALS AND METHODS

Virus. Strain A/PR/8/34 (H0N1) was used in most experiments. Virus was cultivated in embryonated eggs at 35 C, and preliminary treatment was adsorption elution from erythrocytes. Further purification and concentration was obtained by adsorption elution with $BaSO_4$ (6), followed by centrifugation at 50,000 × *g* for 60 min. Virus concentrates were prepared by suspending the pellets in 0.89% sodium chloride with 0.08% sodium azide as preservative.

To extend the determinations of molecular weights by exclusion chromatography to proteins from other virus strains, A/AA/6/60 (H2N2) and the X_7 (F1) recombinant (H0N2) obtained from E. D. Kilbourne (14) were employed in some experiments. For this purpose, virus was pelleted directly from allantoic fluid at 50,000 × *g* for 60 min and then banded on a 5 to 40% tartrate gradient in an SW25 rotor (Spinco) at 50,000 × *g* for 60 min.

Extraction of HABA protein. A 4-ml amount of virus concentrate, with hemagglutinin (HA) titers of the order of 10^6 per ml, was mixed with 15 ml of methanol-chloroform (2:1, v/v) and the mixture was shaken gently by hand for 2 min. Next, 5 ml of chloroform was added, and shaking was continued for 2 min. Then, following addition of 5 ml of water and 2 min of shaking, the mixture was centrifuged at 800 × *g* for 15 min. An insoluble mass, located at the interface, was collected and suspended in 4 ml of water, and a stream of nitrogen was bubbled through the coarse suspension to remove organic solvents. The suspension was centrifuged at 800 × *g* for 15 min, and the supernatant fluid was discarded. The pellet was

either lyophilized for future extraction or immediately suspended in 4 ml of solvent containing 7 M guanidine, 0.05 M dithiothreitol (DTT), and 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (Tris-hydrochloride) (*p*H 7.8), and stirred at room temperature for 7 or 16 hr, during which time the suspension became essentially clear. On dialysis in the cold against PBS (0.01 M phosphate buffer [*p*H 7.4] and 0.1 M NaCl), insoluble proteins flocculated from solution. Dialysis was continued for 48 hr against three changes of PBS, 1 liter each. Preparations were clarified twice by centrifugation at 50,000 × *g* for 1 hr. The clear supernatant fluid is the HABA protein preparation referred to in this presentation. The sediment was either discarded or, if used for analysis, was washed twice by suspension in 30 ml of PBS followed by centrifugation at 50,000 × *g* for 1 hr.

Reagents. Materials and their suppliers were as follows: guanidine HCl, Ultra Pure, and Tris-hydrochloride, Schwarz/Mann, Orangeburg, N.J.; crystallized bovine plasma albumin, Metrix, Armour Pharmaceutical Co., Chicago, Ill.; dithiothreitol, ovalbumin, and bovine gamma globulin, Nutritional Biochemical Corp., Cleveland, Ohio; trypsin and chymotrypsin, Worthington Biochemical Corp.; polyacrylamide gel materials, Bio-Rad Laboratories, Richmond, Calif.

Protein determinations. Protein was estimated by the method of Lowry et al. (18) with bovine serum albumin as standard.

Carbohydrate determinations. The anthrone method of Roe was employed with controls for charring and tryptophan (22). Incubation was at 80 C, and absorption was measured over the spectral range from 500 to 640 nm.

Amino acid analysis. Samples of protein were hydrolyzed with 5.8 N HCl in vacuo after repeated flushing with nitrogen at 110 ± 1 C, and after 24 or 48 hr HCl was removed in vacuo. Amino acid composition was determined with a Spinco model 120 automatic recording analyzer, equipped with an electronic integrator.

Molecular exclusion chromatography. Columns had diameters of 2.5 cm and lengths of 45 cm and were equipped with flow adaptors (Pharmacia Fine Chemicals). Eluant was run through 40 cm of gel by upward flow with a Delta micro-metering pump (Watson-Marlow, Falmouth, England) at a rate of 12 ml/hr, and fractions were collected in 4-ml volumes.

For determinations in saline, Sephadex G150 and G200 were equilibrated with eluant: 0.1 M KCl, 0.05 M Tris-hydrochloride (*p*H 7.8), and sodium azide, 0.08%. Void volumes were determined with Blue Dextran 2,000 and internal volumes with 2,4-dinitrophenol (DNP)-alanine. Protein samples containing 1 to 2 mg were diluted in or dialyzed against eluant. Elution volumes were determined by spectrophotometric measurements of the fractions at 280 nm.

For chromatography in the presence of guanidine, large-pore agarose (Bio-Gel A-50m) was equilibrated against eluant consisting of 6 M guanidine, 0.01 M DTT, 0.1 M KCl, 0.05 M Tris-hydrochloride (*p*H 7.8), and 0.01 M ethylenediaminetetraacetic acid (EDTA). Void volumes were determined with glutaraldehyde-fixed *E. coli* suspensions and internal volumes with DNP-alanine. Protein samples containing 1 to 2 mg were dissolved in eluant in which the guanidine concentration was increased to 7 M. Effluent fractions were analyzed by ultraviolet (UV) absorption at 280 nm and then dialyzed against PBS, the UV absorption was rechecked, and the blocking antigen titers were measured.

Calculation of internal volumes, distribution coefficients, and Stokes radii followed the procedure of Andrews (1). Preparations of HABA protein gave two estimates of elution volumes, one based on blocking antigen titers and the other on UV absorption. The latter values, since they could be estimated with greater precision, were employed to determine distribution coefficients and molecular weights.

Polyacrylamide gel electrophoresis. Formulation of the 7.5% acrylamide-0.1% sodium dodecyl sulfate (SDS) gel system followed the methods of Maizel et al. (19). Samples were dissolved in solvent containing 1% SDS and 1% DTT and were boiled for 2 min. Short-term electrophoresis was in 0.5 by 6 cm gels at 3 mA per gel for 7 hr, and longer runs were in 0.5 by 11 cm gels at 3 mA per gel for 18 hr. Gels were fixed in 50% trichloroacetic acid for 25 hr and then held 8 hr in a solvent containing 45.4% (v/v) methanol and 9.2% acetic acid (w/v) in water. Overnight staining was with 0.25% Coomassie blue in the latter solvent. Gels were destained with two charges of the same solvent, followed by several changes of 7.5% acetic acid in water (w/v). Densitometer tracings were made with the acrylamide gel accessory of a Beckman Acta spectrophotometer.

Antisera. Antisera to whole virus were obtained by intravenous inoculation of rabbits with 0.5 ml of virus concentrate containing 4,000 HA units of virus treated with Formalin (1:4,000). Inoculation was repeated after 1 wk, and the rabbits were bled from the ear vein 1 wk later. Booster doses were given at longer intervals, and animals were again bled 1 wk after booster.

HA and HI tests. Plastic trays were used with reaction mixtures containing 0.2 ml of hemagglutinin, 0.2 ml of antiserum or PBS, and 0.4 ml of 0.5% chicken red blood cells. Each test was carried out in a conventional manner (21).

Blocking antigen test. Stock virus was standardized to 4 HA units per 0.2 ml and antiviral serum to 4 HI units per 0.2 ml. To 0.2 ml of successive twofold dilutions of protein was added 0.2 ml of antiserum. After 30 min at room temperature, 0.2 ml of virus was added to each reaction mixture. After an additional 30 min, during which virus could react with unbound antibody, each cup received 0.2 ml of 1% chicken erythrocytes. Titers were read as the highest dilution of protein giving a positive HA reaction (7).

Immunoaffinity columns. Antisera were first chromatographed on diethylaminoethyl (DEAE)-cellulose to remove HA inhibitor (17). Anti-PR8

serum was dialyzed against 0.017 M phosphate buffer (*p*H 6.3) and clarified at 800 × *g* for 10 min. DEAE-cellulose, (DE-52, Microgranular preswollen, Whatman) was equilibrated against the same solvent and poured as a 1.2 by 27 cm column with an overlay of G-25 Sephadex. A 3-ml amount of antiserum, followed by 0.017 M phosphate buffer eluant, was run through the column by gravity, and 4-ml fractions were collected. Concentrated reagent was then added to each chromatographic fraction to give an NaCl concentration of 0.1 M, bovine serum albumin to 0.5% and sodium azide to 0.08%. HI levels of specific antibody of the fractions were measured with PR8 virus and with A/Japan/305/57 (H_2N_2) heated at 56 C for 30 min as indicator virus for nonspecific inhibitor titers. HI titers for PR8 virus reached 256 at the peak, whereas the corresponding inhibitor titer was less than 2.

Preparation of HABA immunoadsorbent was based on the method of Axen (2, 5). Sepharose 4B (Pharmacia Fine Chemical) was first equilibrated with water. To 100 ml of Sepharose gel, suspended in 100 ml of water, was added 10 g of cyanogen bromide dissolved in 100 ml of water with rapid mixing by using a magnetic stirrer. The *p*H was immediately adjusted with 4 M NaOH and maintained at *p*H 11.0 to 11.3. When the *p*H stabilized, the gel was washed on a Buchner funnel over Whatman no. 1 filter paper, with a total of 2 liters of 0.1 M sodium bicarbonate. Gel was suspended in 200 ml of 0.1 M sodium bicarbonate adjusted to *p*H 9 with sodium hydroxide. A 1-ml amount of HABA protein, containing approximately 1.5 mg of protein, was added, and the mixture was stirred gently overnight at 4 C. The gel was washed exhaustively by successive settling and decanting with a total of 8 liters of PBS containing 0.08% sodium azide.

Chromatography was performed in a 1.2 by 10 cm column of HABA-Sepharose gel by gravity feed. A 1-ml amount of DEAE-cellulose-fractionated antiserum followed by PBS eluant was run through the column at a rate of 15 ml/hr, and twelve 4-ml fractions were collected. The column was washed with an additional 30 ml, and then the eluant was changed to 6 M potassium iodide (KI) to elute adsorbed antibody. The fractions were dialyzed overnight against PBS. Both PBS and KI eluant fractions were then titered for HI antibodies.

Equilibrium filtration. The procedure of Fazekas de St. Groth (10) was adopted to compare antibody bound by virus and protein preparations. Graded dilutions of virus or protein in 1-ml volumes were mixed with 1 ml of rabbit antivirus serum diluted 1:25 with 1% bovine serum albumin in PBS. After 30 min at room temperature, the mixtures were passed through 0.05-μm pore size membrane filters (Millipore Corp.) by positive pressure. Samples were tested to measure the HI titers of the filtrates, and titers of bound antibody were plotted against dilution of antigen. Parallel, best-fitting lines were drawn for each antigen. The regression line for virus was taken as standard, and the distance between the standard line and the lines for each of the other antigens was used to determine the antibody-binding ratios.

RESULTS

Preparation and properties of HABA protein. The procedure for the preparation of HABA protein described under Materials and Methods leads to a separation of viral proteins based on their relative solubility in PBS after lipid extraction and dissociation with guanidine-DTT. The saline-soluble fraction contains the serologically active HABA protein, whereas a major portion of viral protein is in the form of a flocculent precipitate removable by centrifugation. To determine partitioning of viral constituents, protein and hexose content and selected serological activities were determined for HABA preparations, for insoluble sediments, and for original virus concentrates. Four preparations derived from three different concentrates of strain PR8 influenza virus were analyzed.

As shown in Table 1, 15% of total viral protein was found in HABA protein preparations. While the major portion of protein was recovered in the sediment, the total of the two fractions was approximately 10% less than the estimate for virus concentrate. In explanation, while a substance positive in the Lowry test might have been lost on lipid extraction, it seems more likely that clumps of the coarse sediment adhered to the wall while samples were pipetted.

Determinations by the anthrone test gave a value of 0.154 mg of hexose per ml for HABA protein whereas that of the sediment was only 0.139, demonstrating that more than half of the protein-bound hexose was associated with HABA protein (Table 1). Since the latter had a much lower protein content, the percentage of hexose per unit of protein averaged 11.7 for

TABLE 1. *Chemical and serological properties of HABA protein, sediment, and original virus concentrate*[a]

Determination	Virus concentrate	HABA protein	Sediment
Protein, mg/ml	8.76	1.32	6.04
% Protein, HABA/virus		15.1 ± 1.0	
Hexose, mg/ml		0.154	0.139
% Hexose/protein		11.7 ± 1.4	2.3 ± 0.5
HA units/ml	1,024,000	<50	<50
Blocking antigen units/ml		22,000	<50

[a] Average of four preperations.

HABA protein whereas that of the sediment was 2.3%. The lower value for the sediment arises from its being a mixture of proteins, only some of which are glycoproteins. The initial extraction of virus concentrates with methanol-chloroform-water should have eliminated glycolipids as a contributor.

With respect to biological properties, while the original virus concentrates averaged 1,024,000 HA units per ml, neither the HABA fraction nor the sediment had measurable hemagglutinating activity. However, serological activity was recovered. The soluble fraction bound hemagglutination-inhibiting antibodies to an average titer of 22,000 in the HA blocking antigen test, whereas the sediment had no measurable activity (Table 1).

Molecular exclusion chromatography of HABA protein in saline. HABA protein preparations were chromatographed through Sephadex G200 and G150 gel columns in saline eluant to determine purity and physical dimensions of the protein.

Preliminary runs gave asymmetric peaks of UV absorption as well as considerable variation in elution volumes, suggesting that some of the preparations were aggregated (24). To overcome these inconsistencies, systematic studies were made of the effects of storage and protein concentration on the physical state of HABA protein. The data presented in Fig. 1 compare the findings with a standard fresh preparation of HABA protein, with protein stored at 4 C for 4 wk and with a fresh sample prepared in a small volume at 20 times the usual protein concentration, subsequently diluted to 1 to 2 mg/ml just prior to chromatography. The standard preparation gave a single symmetric peak, stored protein gave a peak with a marked shoulder, whereas concentrated protein, even without storage, gave two peaks suggesting dimer formation. On the basis of these results, freshly prepared HABA protein was used for all subsequent physical measurements, and protein concentrations were held below 2 mg/ml during preparative procedures.

A 2-mg amount of HABA protein (Fig. 2) was chromatographed on Sephadex G200 with saline eluant, and the fractions were analyzed for protein by UV absorption and for antigen by blocking antigen tests. A single sharp symmetric peak of protein absorption was centered about fraction 29. Blocking antigen tests gave a similar peak located at the same elution volume. The results demonstrate that only a single size of protein component is present in HABA protein preparations and that this protein is the antigen. In four of twenty

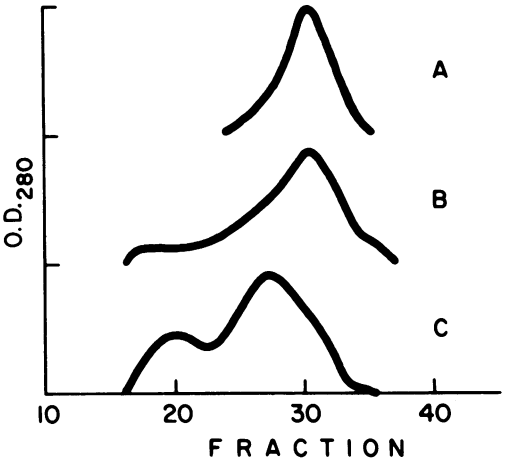

FIG. 1. *Demonstration of aggregation of HABA protein on long-term storage and on preparation at high protein concentration. Samples were chromatographed on Sephadex G-200 with saline eluent consisting of 0.1 M KCl, 0.05 M Tris-hydrochloride (pH 7.8), and 0.8% sodium azide, and protein content of effluent fractions was measured by absorption at 280 nm. A, Standard HABA protein sample prepared at about 2 mg of protein per ml and tested immediately after preparation. B, Similar preparation stored for 4 wk at 4 C. C, Sample prepared in small volume containing 40 mg of protein per ml, diluted to 2 mg per ml after preparation, and immediately chromatographed.*

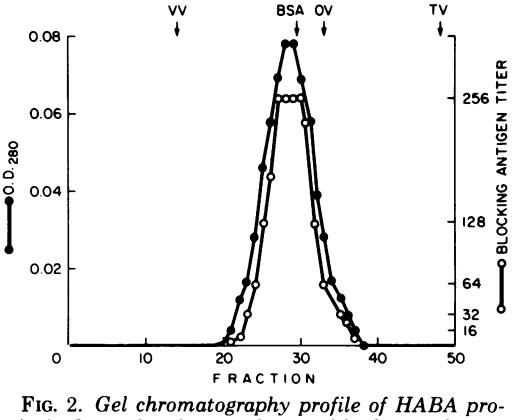

FIG. 2. *Gel chromatography profile of HABA protein in Sephadex G-200 column with eluant of 0.1 M KCl, 0.05 M Tris-hydrochloride (pH 7.8), and 0.08% sodium azide. Fractions were tested for both protein content (closed circles) and blocking antigen activity (open circles). Reference markers: void volume (VV), bovine serum albumin (BSA), ovalbumin (OV), and total internal volume (TV).*

preparations a minor second peak, located at the void volume, was detected. This secondary peak represents either aggregated HABA or high-molecular-weight protein, but on anal-

ysis never exceeded values of 3% of total protein content or blocking antigen activity.

To determine the molecular dimensions of HABA protein, a Sephadex G200 column was calibrated with four reference proteins to establish the slope of a linear relationship between distribution coefficient and logarithm of molecular weight. Five preparations of HABA protein, three prepared from influenza strain PR8 and one each from recombinant X7(F1) and strain AA/6/60, were then run through the column, their elution volumes were measured, and distribution coefficients were calculated (Table 2). Molecular weights were determined by interpolation on the standard plot of the distribution coefficients of the HABA protein samples (Fig. 3). The average molecular weight was estimated at 78,000. The value for X7(F1) showed the largest deviation from the mean, but a measurement in another run, with Sephadex G150, gave a value of 74,000 in molecular weight. Calculations of the Stokes radius of HABA protein averaged 37.8.

Molecular exclusion chromatography of HABA polypeptide subunits in guanidine solvent. Previously published results (7) demonstrated that HABA protein in saline had a sedimentation constant of 4*s*, while in dissociating reagents, such as urea or guanidine, this was reduced to 2*s*, suggesting that the antigen consisted of two, or possibly three, polypeptide subunits. To determine the number and size of polypeptides, HABA protein was dissociated in 7 M guanidine with 0.05 M DTT, and the samples were chromatographed through a Bio-Gel A50 agarose column with guanidine solvent as eluant. The effluent fractions were tested for polypeptide by UV absorption and yielded a single symmetrical peak centered about fraction 35 (Fig. 4). The results indicate that only a single size of polypeptide is derived from HABA protein. The fractions were then dialyzed against PBS to remove guanidine and DTT and permit reassociation of the polypeptides to yield serologically active HABA protein. The dialyzed fractions were assayed for blocking antigen titers, and all activity was localized in the same fractions as the polypeptide peak (Fig. 4).

TABLE 2. *Exclusion chromatography of HABA protein in saline on Sephadex G200*

Determination	Distribution coefficient	Molecular weight	Stokes radius
Gamma globulin	0.309	205,000[a]	53
Bovine serum albumin	0.488	69,000	35.5
Ovalbumin	0.568	42,500	27.3
Chymotrypsin	0.677	22,500	22.4
HABA PR8 (1)	0.476	75,000	36.2
(2)	0.456	84,000	39.5
(3)	0.462	82,000	38.8
AA/6/60	0.462	82,000	38.8
X7 (F1)	0.493	68,000	35.5
HABA protein average	0.470	78,000	37.8

[a] Effective molecular weight.

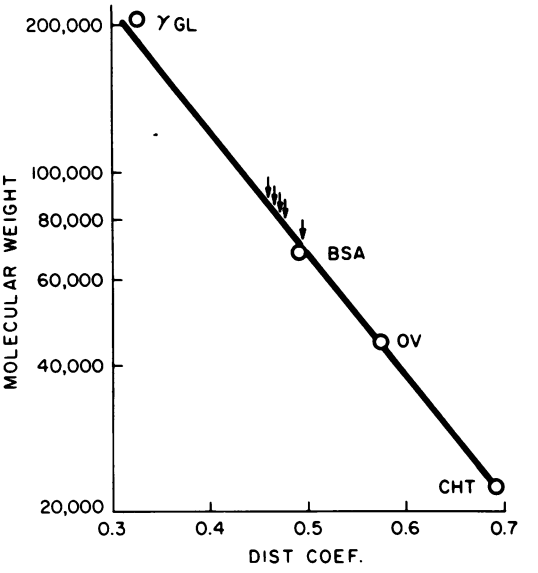

FIG. 3. *Estimation of molecular weight of HABA protein by gel chromatography by using procedure described in Fig. 2. Slope of linear relation between distribution coefficients and logarithms of molecular weights was determined with four reference proteins: bovine immunoglobulin G (γGL), bovine serum albumin (BSA), ovalbumin (OV), and chymotrypsin (CHT). The five arrows refer to experimentally determined distribution coefficients of HABA protein preparations.*

Quantitative estimates of dimensions of HABA polypeptide were obtained by calibrating the column with reference proteins (Table 3). Five preparations of HABA protein obtained from influenza virus strain PR8 were tested, and an average molecular weight of 39,000 was obtained (Fig. 5). Coupled with the molecular weight value of 78,000 for HABA protein in saline, the determinations indicate that two polypeptide subunits, apparently identical in size, combine on removal of dissociating reagents to form the serologically active HABA molecule.

Acrylamide gel electrophoresis. When HABA protein was dissociated into polypeptide subunits with 1% SDS and DTT and

subjected to electrophoresis through 7.5% acrylamide gel, only a single major band was observed (Fig. 6A). On comparison with marker proteins, HABA polypeptide was found to migrate at about the same rate as ovalbumin. Calculations of molecular weight by interpolation gave estimates ranging from 42,000 to 45,000, values slightly higher than determinations by molecular exclusion chromatography. In some preparations, a faint band was detected in the region of molecular weight 80,000. This might correspond to the HA band of SDS-dissociated virus or result from failure to dissociate, or partial aggregation of, HABA polypeptides. In other preparations, a faint band was found in the region of molecular weight 25,000. Since this latter band might indicate some contamination with HA_2, the other polypeptide associated with the hemagglutinin spike, a careful search of all HABA preparations subjected to electrophoresis was made with a densitometer. In 4 out of 11 gels, a faint band was found in this range. The example used in Fig. 6A had the most obvious band and was quantitatively analyzed by densitometry to obtain an estimate of maximum HA_2 contamination of HABA protein. The areas under the two peaks were compared, and the value for the minor one was 4% of that of the major peak.

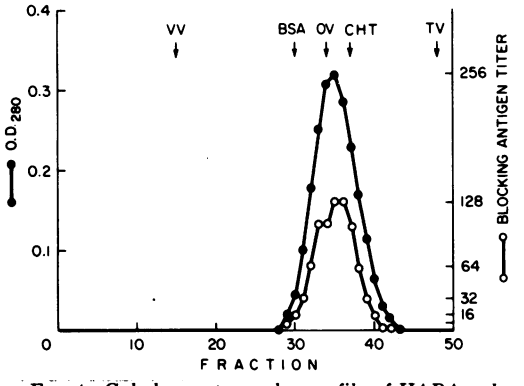

FIG. 4. *Gel chromatography profile of HABA polypeptide in Bio-Gel A50 agarose column with eluant: 6 M guanidine, 0.1 M KCl, 0.01 M EDTA, 0.5 M Tris-hydrochloride (pH 7.8), and 0.01 M DTT. Effluent fractions were tested for protein content (closed circles) and after dialysis against PBS for blocking antigen activity (open circles). Reference markers: bovine serum albumin (BSA), ovalbumin (OV), and chymotrypsin (CHT).*

TABLE 3. *Exclusion chromatography of PR8 strain HABA protein in guanidine solvent on Bio-Gel A50*

Sample	Distribution coefficient	Molecular weight	Stokes radius
Bovine serum albumin	0.476	69,000	35.5
Ovalbumin	0.557	42,500	27.3
Chymotrypsin	0.641	25,000	22.4
HABA protein (1)	0.583	35,500	25.6
(2)	0.588	35,000	25.6
(3)	0.557	42,000	27.0
(4)	0.577	37,500	26.0
(5)	0.553	42,500	27.3
HABA protein average	0.568	39,000	26.3

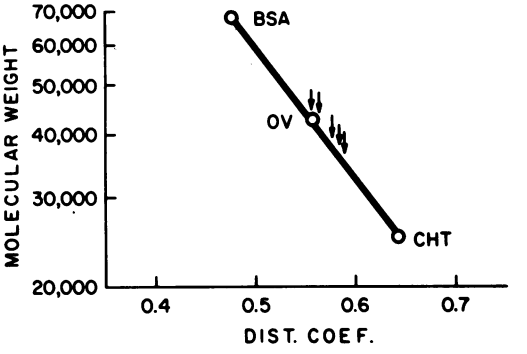

FIG. 5. *Estimation of molecular weight of HABA polypeptide by using procedure described in Fig. 4. Slope of linear relationship between distribution coefficients and logarithms of molecular weights was determined with three reference proteins: bovine serum albumin (BSA), ovalbumin (OV), and chymotrypsin (CHT). The five arrows refer to experimentally determined distribution coefficients of HABA polypeptide preparations.*

To document further the degree of purity of HABA protein, comparisons were made of the composition of HABA protein, of insoluble sediment, and of original virus concentrate treated in the same manner with SDS and DTT prior to electrophoresis. The findings are also shown in Fig. 6. In accordance with the numbering system proposed by Kilbourne et al. (13), three faint bands, P_1, P_2, and HA, were regularly detected in the virus sample near the origin, but were not found in the HABA preparation. A first major peak in the region of molecular weight 60,000, containing the closely spaced NP and NA polypeptides, was found in the sediment and virus samples, but again was not present in the HABA sample. The second major peak, HA_1, corresponds to the single major peak of HABA protein, is present in the virus sample, and is absent in the sediment. A slight asymmetry at the base of this peak may indicate traces of HA_1 remain-

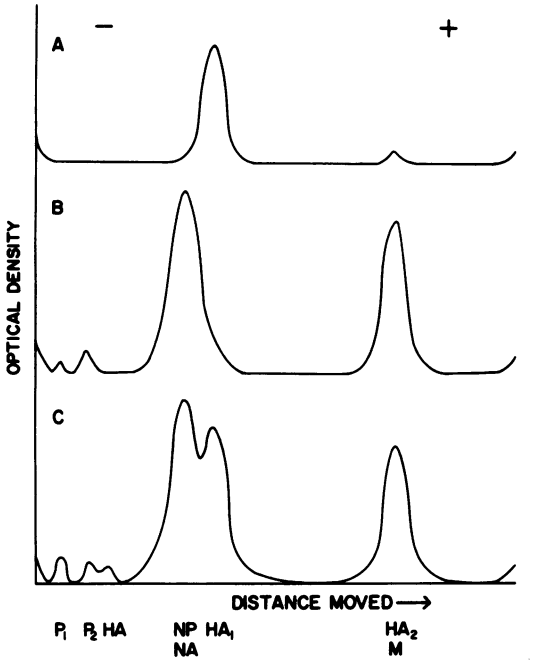

FIG. 6. *Densitometer profiles of acrylamide gel electrophoresis patterns of (A) HABA protein, (B) insoluble residue from HABA protein preparation, and (C) whole virus. Lyophilized samples were dissolved in 1% SDS with 1% DTT, and subjected to electrophoresis in 11-cm-long gels containing 7.5% acrylamide and 0.1% SDS for 18 hr at 3 mA per gel. Gels were stained with Coomassie blue, and optical density scannings were read at 492 nm. Letters at the bottom of the figure refer to proposed influenza polypeptide nomenclature (13). Observed base lines of B and C were at an angle to horizontal axis, and above curves were redrawn by using optical density values calculated from differences between observed values and base line values. Curve A was not adjusted since base line was horizontal.*

ing in the sediment. Finally, both sediment and virus samples gave major peaks in the region of molecular weight 25,000 (HA_2 and M proteins), whereas HABA protein contained only a trace. The data provide explicit evidence that HABA protein is a highly purified preparation of HA_1 polypeptide and that essentially all HA_1 is extracted and then recovered in HABA protein preparations.

Amino acid composition of HABA protein. Published data on the amino acid composition of influenza virus proteins are available for neuraminidase (12) and the heavy and light chains of hemagglutinin (15) which probably correspond to HA_1 and HA_2 polypeptides, respectively. The amino acid composition of HABA protein prepared from PR8 strain of influenza virus (Table 4) differs decisively from that of neuraminidase, although it should be pointed out that the enzyme is derived from a different strain of virus, X7(F1) recombinant. In contrast, there is a marked resemblance between the composition of HABA protein of PR8 strain and both the light and heavy chains from A/BEL strain, major exceptions being in the estimates of phenylalanine and methionine, the latter of which would be influenced by oxidation. Further, the single major difference between light and heavy chains is in the amount of proline (15). HABA protein and heavy chain both have higher proline contents, providing further evidence that HABA protein is related to heavy chain or HA_1 polypeptide.

Adsorption of HI antibodies to HABA protein affinity columns. Although the hemagglutinin spike contains two kinds of polypeptides, HA_1 and HA_2, it is possible that only HA_1 is directly involved in HA antigenicity. Apparently complete binding of antibody to HABA protein in blocking antigen tests suggested that this was so, but direct evidence in a more sensitive test was sought by using immunoaffinity columns. HABA protein was attached to a cyanogen bromide-treated Sepharose matrix, and a chromatographic column was prepared. Antiviral serum, fractionated on DEAE-cellulose to remove inhibitor, was run through the column with PBS as eluant. As shown in Table 5, no HI antibody

TABLE 4. *Amino acid composition of HABA protein compared with heavy and light chain*

Amino acid	HABA protein		Malar ratios[a]	
	Residues/ polypeptide	Molar ratios	Heavy chain	Light chain
Lysine	22.6	0.498	0.50	0.57
Histidine	18.6	0.186	0.17	0.12
Arginine	34.8	0.348	0.37	0.19
Aspartate	45.4	1.000	1.00	1.00
Threonine	20.8	0.459	0.62	0.32
Serine	38.1	0.838	0.79	0.56
Glutamate	39.0	0.859	0.97	0.96
Proline	25.4	0.559	0.39	0.04
Glycine	27.7	0.609	0.73	0.76
Alanine	18.1	0.398	0.47	0.40
Valine	20.7	0.455	0.52	0.43
Methionine	7.3	0.160	0.04	0.06
Isoleucine	20.2	0.444	0.56	0.43
Leucine	31.7	0.697	0.76	0.69
Tyrosine	11.1	0.245	0.29	0.26
Phenylalanine	5.8	0.128	0.28	0.31
Cysteine	9.2	0.202	0.13	0.08

[a] Adapted from the data of Table 1 in reference 15.

TABLE 5. *Fractionation of anti-PR8 influenza antibodies on immunoaffinity column of PR8 HABA protein-Sepharose*

Fraction	HI Titer	
	PBS	KI
1	$<2^1$	$<2^1$
2	$<2^1$	$<2^1$
3	$<2^1$	2^2
4	$<2^1$	2^6
5	$<2^1$	2^5
6	$<2^1$	2^3
7	$<2^1$	2^4
8	$<2^1$	2^4
9	$<2^1$	2^2

was detected in effluent fractions, indicating that HI antibodies were completely adsorbed by HABA protein. To dissociate the antigen-antibody complexes, 6 M KI was used as eluant. Released antibodies in the KI fractions totaled 360 HI units whereas the input was 512 HI units, a recovery within experimental error. The findings demonstrate that HI antibodies induced by whole virus are primarily oriented to HABA protein or to HA_1 polypeptide.

Antibody binding capacity of HABA protein. In the course of the preparation of HABA protein, the HA_1 polypeptides would undergo conformational changes, and it is possible that antigenic determinants would be blocked or alternatively freed from steric hindrance imposed by the structure of the spike. To investigate these possibilities, the HI antibody binding capacities of HABA protein preparations were compared with those of the original virus concentrates employing equilibrium filtration techniques. Samples (4-ml) of virus concentrates were converted into HABA preparations to give the same 4-ml volume. Graded dilutions of virus or protein were incubated with constant amounts of antiviral serum and then filtered, and the filtrates were tested for residual HI titers. As shown in Table 6, virus concentrate at a dilution of 1:100 reduced the HI titer from the control value of 2^6 to 2^2. At the same dilution, HABA protein removed all detectable antibody. On comparison of virus and HABA protein at the several dilutions, the HABA protein in each case bound more antibody than intact virus. Five preparations were compared, and the ratios of HI antibody bound by HABA protein to that bound by virus ranged from one- to twofold, averaging 1.7-fold. The results indicate that about twice as many HI antigenic sites are available on HABA protein as compared to the intact spike. Since HABA protein preparations account for only 15% of total viral protein, they were estimated on a nitrogen basis to be 11 times more active serologically than intact virus.

Immune response to HABA protein. Although the antigenicity of HABA protein has been demonstrated in serological tests, it was important to determine whether it is a complete antigen and induces antibodies in experimental animals. HABA protein from strain PR8 influenza virus was inoculated into a group of eight mice. The first dose, containing 0.26 mg of protein suspended in Arlacel-mineral oil adjuvant, was inoculated intraperitoneally. Ten weeks later, each mouse received a booster dose of 0.26 mg without adjuvant. Mice were bled 10 days later, and sera were tested for HI antibodies. Titers ranged from 64 to 2,048, averaging 1,400. In egg neutralization tests, the titers averaged 1:40.

With respect to the possibility that anti-HABA protein serum contains antibodies to both HA_1 and HA_2 components of hemagglutinin, direct demonstration of absence of antibodies to HA_2 polypeptide was not feasible due to lack of definitive HA_2 reagents. To reduce possible antibody induction by trace contaminants of HA_2 polypeptide, HABA protein chromatographically fractionated on Sephadex G200 was employed as inoculum. No HA_2 band was detected on polyacrylamide gel electrophoresis of this preparation. Further, whereas large dosages of protein are essential to induce significant HI antibody titers, the amount of protein inoculum employed was minimal to achieve such titers. Unless HA_2 polypeptide has a markedly greater antigenic potential than HA_1, the antiserum to HABA

TABLE 6. *Comparison of HI antibody binding by virus and HABA protein as determined by equilibrium filtration*

Antigen	Antigen dilution	HI titration							
		2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8
Virus	1:100	0	0	3	3	3	3	3	3
	200	0	0	0	0	1	3	3	3
	300	0	0	0	0	0	1	3	3
	400	0	0	0	0	0	0	3	3
	600	0	0	0	0	0	0	3	3
HABA protein	1:100	3	3	3	3	3	3	3	3
	200	0	2	3	3	3	3	3	3
	300	0	0	0	1	3	3	3	3
	400	0	0	0	0	0	2	3	3
	600	0	0	0	0	0	0	3	3
Serum control		0	0	0	0	0	0	3	3

protein can be expected to possess a high degree of specificity for HA_1 polypeptide.

DISCUSSION

The low-molecular-weight HABA glycoprotein extracted from virus hemagglutinin with either concentrated urea or guanidine, in the presence of reducing agents, has three important characteristics: physical homogeneity, high degree of purification, and immunological activity. Homogeneity of HABA protein has been documented by rate zonal (7) and isopycnic centrifugation (8), immunodiffusion (8), and in the present report by molecular exclusion chromatography. In like manner, data based on acrylamide gel electrophoresis (9), analytical ultracentrifugation (8), and molecular exclusion chromatography all agree on the physical uniformity of HABA polypeptides. The physical-chemical data project only a single size of polypeptide with a molecular weight of the order of 40,000 and a HABA protein molecule of 78,000. This suggests that, on removal of dissociating reagents, there is a spontaneous association of two identical polypeptides or, alternatively, two different polypeptides of similar size to form the HABA molecule. The data also provide evidence of the high degree of purity of the product obtained by the HABA protein fractionation procedure.

Despite some discrepancies in quantitative estimates of size, HABA polypeptides appear to be related to the HA_1 polypeptides of SDS-dissociated influenza virus (13). The estimate of molecular weight 39,000 obtained by exclusion chromatography is lower than the value of 47,000 obtained by Webster (24). On acrylamide gel electrophoresis of HABA protein, the molecular weight estimate is 42,000 to 45,000 as compared to published values of 47,000 to 50,000 for HA_1 polypeptide (4). Yet, in terms of percentage of total viral protein, glycoprotein nature, and association with hemagglutinin, HABA and HA_1 polypeptides appear to be closely related if not identical. The discrepancies reported may be due to variations inherent in the technical procedures or there may be real differences in the polypeptides, particularly their saccharide moieties, arising from the use of different virus strains, host cells, and preparative procedures.

The results of antigenic studies of HABA protein have a bearing on the respective roles of HA_1 and HA_2 polypeptides in the structure and immunology of the hemagglutinin spike. Chromatography of antiviral serum in immunoaffinity columns of HABA protein leads to adsorption of all HI antibodies to HABA protein. On the basis of this evidence, it is concluded that, while the second polypeptide, HA_2, is spike associated, it has a structural function but is not directly involved with those sites involved in hemagglutination (3, 13). It is possible, however, but improbable on theoretical grounds, that the two dissimilar polypeptides share antigenic determinants. Complete definition of the functions of HA_1 and HA_2 glycopeptides awaits isolation of HA_2 polypeptide in a comparable state of purity and antigenic activity.

Evidence of the organization of HA_1 on the HA spike has been obtained by equilibrium filtration studies which indicate that approximately twice as many antigenic sites are available on HABA protein as compared to intact spike. A released HABA molecule may react with two antibody molecules, whereas, when restrained by the spike structure, only a single site, corresponding to one HA_1 polypeptide, is exposed at the tip. Another explanation is that steric limitations determine the number of antibody molecules capable of attachment to the virus surface, i.e., the virion has more antigenic sites on its surface than can be packed with antibody molecules. On this basis, dispersion of HABA protein as a soluble antigen would allow all sites to react.

The data permit formulation of a skeletal model of a hemagglutinin spike. Two HA_2 polypeptides would constitute the inner stalk of the spike, whereas two HA_1 polypeptides would form the head. On such a basis the molecular weight of a spike would be 130,000, a value close to that of 150,000 estimated by electron microscopy (15).

The hemagglutinin product bearing the closest resemblance to HABA protein is the heavy chain isolated by Laver (15) by using successive SDS and guanidine-DTT extractions. Chemical data based on solubility and amino acid composition demonstrate the similarity of the two protein preparations, but they differ in that heavy chain preparations have essentially no serological activity. One possible explanation for this loss of antigenicity is that the chains are restricted to altered conformations by residual, bound SDS, since there is evidence that dialysis is not sufficient to remove protein-bound SDS but rather extraction with organic solvents is required (20). A second possibility is that the mode of denaturation is a crucial factor in reversible recovery of protein in a biologically active conformation. In comparative studies of renaturation of several enzymes, it has been demonstrated that there is a correlation between reassembly of native

structure and recovery of enzymatic activity (23). Tanford has concluded that SDS and guanidine denaturations of globular proteins lead to different conformations, guanidine to an extended random state and SDS to a rigid rod with a high degree of internal order (11). The latter type of denaturation might preclude realignment or reassociation of polypeptides to a structure similar to the native state. Thus, in the case of SDS-dissociated hemagglutinin substructures (16), hemagglutinating activity would be retained, but regions of the chains would be irreversibly altered by SDS denaturation or adsorption of SDS. On guanidine dissociation of the substructure into light and heavy chains, the SDS-altered chains would not be capable of realignment or recombination to antigenically active conformations (15). In contrast, while the HABA protein extraction procedure also leads to a series of drastic conformational changes related to dissociation of the spike, denaturation to an extended state, and renaturation and reassociation of glycopeptides, a conformation of HABA molecule is reestablished which is identical to that of native protein insofar as can be detected by sensitive serological tests.

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