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Chemical and Immunological Analysis of the Complex Structure of *Escherichia coli* Alpha-Hemolysin

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***Escherichia coli* alpha-hemolysin (AH) purified from culture supernatants by gel filtration and ion-exchange chromatography was heterogeneous in charge and size. A 107,000-dalton protein was identified as the product of the *hlyA* gene by its reactivity with anti-AH monoclonal antibodies. Proteolysis of the product of the *hlyA* gene occurred but was not required for transport of the protein through the cell wall. Active AH had a larger size and lower pI than analysis of the *hlyA* gene sequence would predict, thus suggesting that the *hlyA* protein is complexed with other bacterial products. Lipopolysaccharide was detected in purified hemolysin complex preparations and may be a major component of the complexes. These findings suggest several possible mechanisms for release of AH from the bacterial cell including release by outer membrane fragmentation. The existence of AH complexed with lipopolysaccharide may have important implications in understanding its toxicity.**

Escherichia coli alpha-hemolysin (AH) is a protein that lyses erythrocytes from several species of animals in vitro. AH is of interest for several reasons. First, *E. coli* isolates from extraintestinal infections produce AH more commonly than intestinal strains do (19), and the hemolysin is believed to be a virulence factor. Further evidence for this belief is that partially purified AH preparations possess several toxic biological properties (3–5). Second, AH is one of very few proteins produced by members of the family *Enterobacteriaceae* that is released extracellularly (23). Finally, the genetic control of AH production, transport, and release from the cell is complex. At least four genes located on the bacterial chromosome or on large transmissible plasmids are required to elicit a cell-free hemolytic phenotype (8, 25).

The hemolysin structural gene (*hlyA*) codes for a 106,000- to 110,000-dalton protein that can be detected in the cytoplasm of *E. coli* cells (8, 10). Initially Noegel et al. (20) and others (12) reported that proteolytic cleavage of the product of the *hlyA* gene into a 58,000-dalton protein occurs in the cytoplasm as a prerequisite for activation, transport, and release from the cell. According to this controversial model the 107,000-dalton protein represents a hemolytically inactive precursor protein that is not found outside the cell. In contrast, Mackman and Holland (18) demonstrated a 107,000-dalton protein as a major component of supernatants from cultures of hemolytic *E. coli* cultures. Felmlee et al. (7) demonstrated a 110,000-dalton protein in *E. coli* culture supernatants that contains the N-terminal amino acids predicted by the DNA sequence of the *hlyA* gene. These latter findings contradict the model proposed by Noegel et al. (20) and suggest that cleavage of the *hlyA* protein is not required for an active extracellular hemolysin.

Neither the 58,000-dalton nor the 106,000- to 110,000-dalton putative hemolysin protein has been purified to homogeneity in a hemolytically active form. Denaturing conditions required for demonstration of extracellular *E. coli* proteins by gel electrophoresis inactivate the hemolysin. Measurements have been made of the size of nondenatured active AH by gel filtration chromatography (13), electron microscopy (22), diffusion coefficient determination (22), and membrane ultrafiltration (3). With these techniques active AH has a molecular size in excess of 150,000 to 500,000 daltons. Since the *hlyA* gene is large enough to code for a 107,000- to 110,000-dalton protein it seems likely that the protein exists in complexes in *E. coli* cultures.

This study was undertaken to analyze the chemical composition of active AH. Knowledge of other components in AH complexes is important not only from a structural standpoint but also in assessment of AH toxicity during infections. We report here that highly purified AH complexes consist of numerous proteins, carbohydrates, and lipids, including lipopolysaccharide (LPS). A hemolysin-neutralizing monoclonal antibody enabled us to identify the major extracellular AH protein.

MATERIALS AND METHODS

Organism and culture conditions. All experiments were done with *E. coli* H-79. This strain was a clinical isolate from a human with a urinary tract infection treated at West Virginia University Hospital, Morgantown, W.Va. Biochemical identification was done with the API 20E system (Analytab Products, Plainview, N.Y.). *E. coli* H-79 contains no plasmids detected by routine screening techniques and therefore appears to harbor chromosomal *hly* determinants.

E. coli H-79 was grown in the chemically defined medium described previously (24). The medium (750 ml) was inoculated from an aliquoted stock that was stored frozen at −70°C. Incubation was at 37°C. (200 rpm).

Purification of AH. Culture supernatants were harvested during the late-exponential growth phase (11 h). AH was purified by a combination of ultrafiltration, gel filtration, and ion-exchange chromatography. All procedures were done at 4°C.

(i) Preparation of concentrated crude hemolysin (CH). Bacterial cells were removed by centrifugation (13,700 × *g*) and filtration through a 0.45-μm-pore-size Durapore membrane filter (Millipore Corp., Bedford, Mass.). The filtrate was concentrated approximately 40-fold by ultrafiltration with an XM-300 membrane (Amicon Corp., Danvers, Mass.) as described previously (3) and clarified by ultracentrifugation at 235,000 × *g*.

(ii) Gel filtration chromatography. CH was partially puri-

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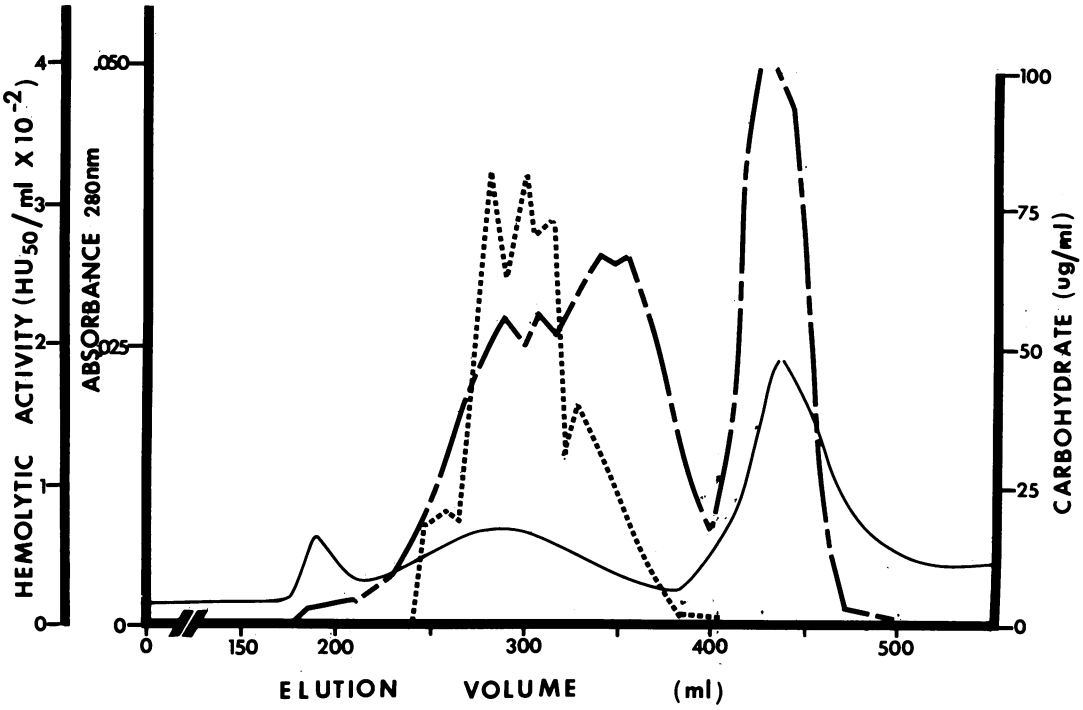


FIG. 1. Fractionation of CH by gel filtration. 9.9 ml of CH (2,800 HU_{50}/ml) was applied to a bed of Sephacryl S-1000 and eluted at 88.8 ml/h (V_0 = 188 ml). Column effluent A_{280} (——) was measured, and fractions were analyzed for carbohydrate (— - —) and hemolytic (- - -) activity.

fied by gel filtration chromatography with an 87- by 2.6-cm bed (bed volume [V_t] = 461.6 ml) of Sephacryl S-1000 (Pharmacia Fine Chemicals, Piscataway, N.J.). The sample (7 to 10 ml) was applied to the top of the gel and eluted (88 ml/h) with 0.05 M sodium acetate buffer (pH 4.0) (ACB) containing 0.2 M NaCl (ACB–0.2 M NaCl). The void volume (V_0) of the column was determined by measuring the volume of buffer required to elute calf thymus DNA under identical conditions (V_0 = 188 ml). Fractions (8 ml) were collected and dialyzed against saline (0.9% NaCl). Elution of hemolytic activity was characterized by calculation of the k_{av} value (21) for the column conditions used according to the equation: $k_{av} = (V_e - V_0)/(V_t - V_0)$ where V_e is the volume at which peak hemolytic activity elutes.

(iii) Ion-exchange chromatography. Fractions from Sephacryl S-1000 gel filtration with peak hemolytic activity were pooled (40 to 60 ml) and applied to the top of a bed of DEAE-Sephadex A-25 (Pharmacia) (1.5 by 15 cm) equilibrated with ACB–0.2 M NaCl. Hemolytic activity was eluted from the column with sequential additions of ACB–0.3 M NaCl and ACB–0.4 M NaCl. Fractions (7 ml) were collected and dialyzed against saline before analysis.

Assay for hemolytic activity. Hemolytic activity was determined by the method of Snyder and Koch (24) except that 50% lysis was used for the endpoint determination. One HU_{50} is the amount of AH required to lyse 50% of the erythrocytes in 1.0 ml of a 1% sheep erythrocyte suspension.

Chemical determinations. Protein assays were done with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) as the standard. Carbohydrates were quantitated by the phenol-sulfuric acid method with a glucose standard (6).

LPS purification. *E. coli* H-79 was grown in 750-ml volumes of chemically defined medium prepared with pyrogen-free distilled water (Travenol Laboratories, Deerfield, Ill.). The cells were recovered by centrifugation, washed, and lyophilized. A total of 20 g (dry weight) of cells was used for purification of LPS by the procedure of Westphal and Jann (26). A yield of 293 mg (dry weight) of LPS was obtained.

SDS-PAGE. AH samples were concentrated up to 10,000-fold by using a combination of ultrafiltration (XM-300 membranes) and Minicon B-15 concentrators (Amicon Corp.). The concentrates were boiled for 7.5 min in an equal volume of 0.25 M Tris buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue. Electrophoresis was performed at constant current with 15 mA through a 5% polyacrylamide stacking gel (1.5 by 140 by 30 mm) and a vertical slab resolving gel (1.5 by 140 by 100 mm) consisting of a 5 to 15% polyacrylamide gradient. The discontinuous system of Laemmli (16) was employed. Electrophoresed proteins were stained with 0.2% Coomassie blue R-250. All reagents for SDS-polyacrylamide gel electrophoresis (PAGE) including molecular weight protein standards were purchased from Bio-Rad Laboratories.

Immunization of rabbits. Coomassie blue-stained CH protein bands were excised from polyacrylamide gels. Proteins were eluted electrophoretically in a model 1750 ISCO electrophoretic concentrator (Instrumentation Specialities Co., Lincoln, Nebr.) by the method of Caldwell and Schacter (1). Recovered proteins were adjusted to the desired concentration and used for immunization of adult male New Zealand White rabbits. Each rabbit received weekly intramuscular and subcutaneous injections of protein (25 to 60 μg). The first two injections were administered with Freund incomplete adjuvant. After 5 weeks, serum samples were screened for anti-AH activity by direct enzyme-linked immunosorbent assay (ELISA).

Monoclonal antibody production. Except where noted, all

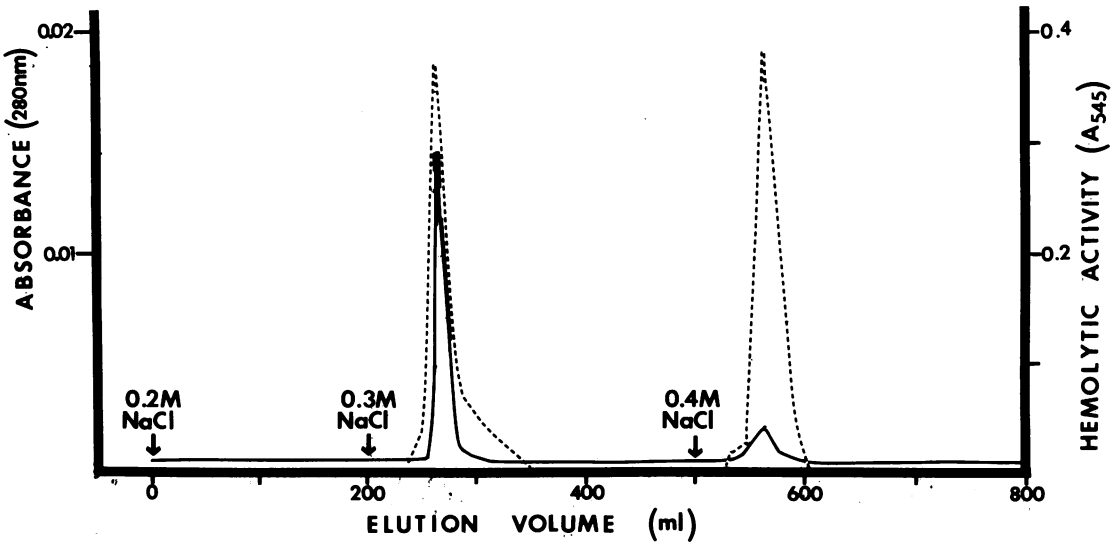


FIG. 2. Fractionation of pooled hemolytic fractions from Sephacryl S-1000 columns by ion-exchange chromatography. 41 ml of sample was applied to a bed of DEAE-Sephadex A-25 with ACB-0.2 M NaCl. Hemolysin was eluted with ACB-0.3 M NaCl and ACB-0.4 M NaCl. The A_{280} (——) of the column effluent was monitored. Relative hemolytic activity (– – –) of column fractions is expressed as A_{545} of supernatant from a standard sheep erythrocyte suspension after incubation with a 1:100 dilution of each fraction.

materials for monoclonal antibody production were purchased from M.A. Bioproducts, Walkersville, Md. Male BALB/c mice (8 weeks old) were used as a source of immune lymphocytes for hybridoma production. For 1 week before immunization, low doses of CH (10 to 100 HU_{50}) were administered daily to each mouse by the intraperitoneal route to induce tolerance to the endotoxin present in the antigen preparation. After tolerization the animals were immunized for 2 months by the multiple injection site technique described by Caterson et al. (2). Each dose of CH antigen consisted of 500 HU_{50} in Freund complete adjuvant (day 1), Freund incomplete adjuvant (day 3), or saline (three times weekly during weeks 2 through 8).

The mice were sacrified 2 days postimmunization by cervical dislocation. Lymphocytes were obtained from their axillary, inguinal, brachial, and popliteal lymph nodes and fused with X63-Ag8.653 mouse myeloma cells (15). The cells were plated in a 96-well plate and grown in the presence of hypoxanthine, aminopterin, and thymidine to select for lymphocyte-myeloma cell hybrids.

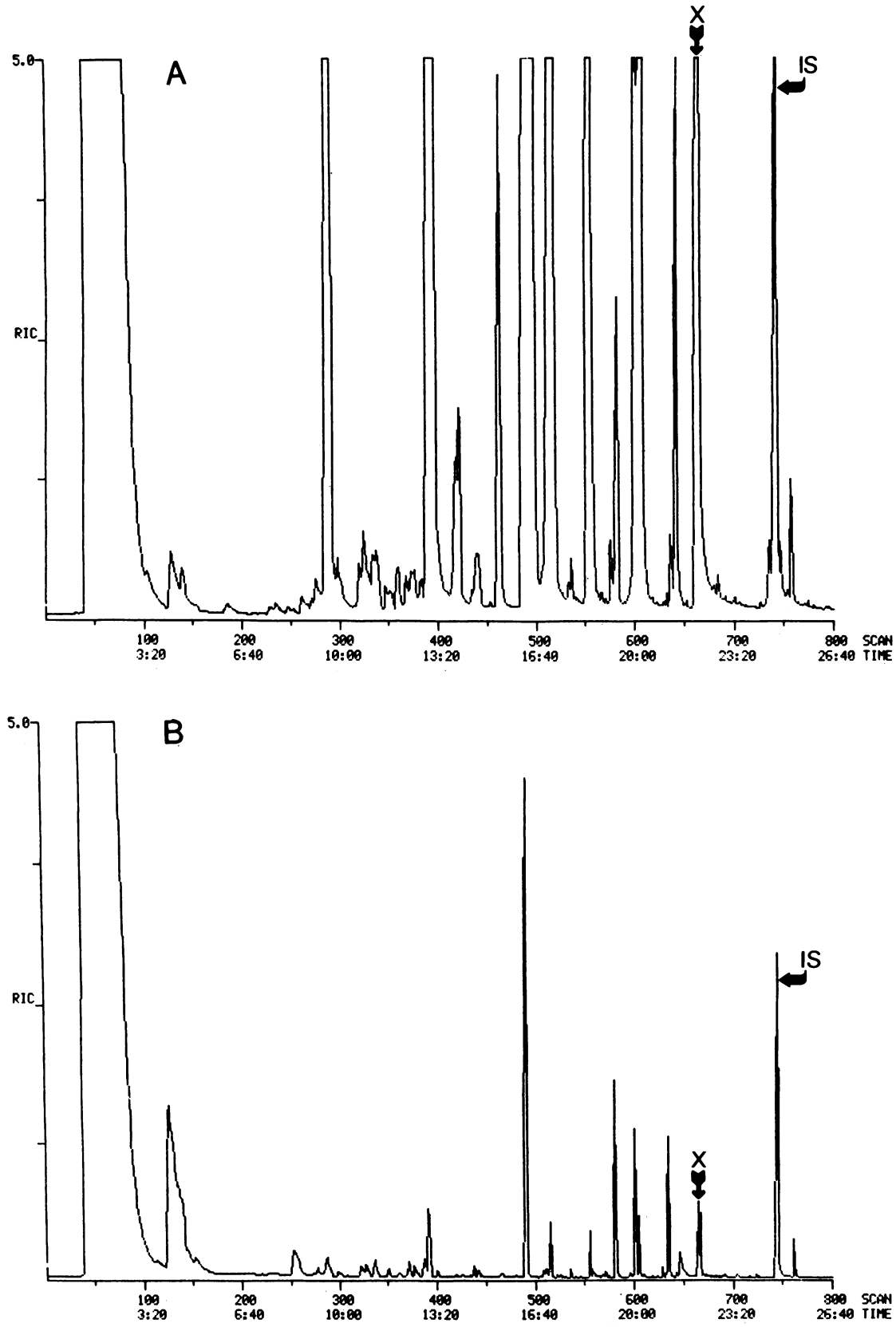
Culture supernatants were screened for antibody by direct ELISA and hemolysin neutralization assays (see below). Cultures that gave negative ELISA reactivity against purified LPS from *E. coli* H-79 but positive ELISA tests with CH or that neutralized the hemolytic activity of a standard CH preparation were cloned twice by limiting dilution in growth medium. Clones were expanded in 260-ml culture flasks. Mice were sensitized with an intraperitoneal injection of pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 24 h later were given an injection containing 10^7 hybridoma cells. After 1 to 2 weeks ascitic fluid was harvested. A control ascites preparation was prepared by injection of mice with X63-Ag8.653 myeloma cells.

ELISA assays. Rabbit sera, hybridoma culture supernatants, and ascites were tested for antibody reactivity by direct ELISA. Wells of polyvinyl assay plates (Costar, Cambridge, Mass.) were coated with test antigen and blocked with BSA as described by Gustafsson et al. (11). Antigen preparations used in this study contained either 25 μg of purified LPS from *E. coli* H-79 or 100 HU_{50} of CH per ml. Reactivity of rabbit polyclonal or mouse monoclonal antibodies with the test antigen was detected with alkaline phosphatase-conjugated goat antibody preparations (Kirkegaard and Perry Laboratories, Inc., Gaithersburg,

TABLE 1. Results of a typical purification run

Step	HU_{50}/ml	Carbohydrate (μg/ml)	Protein (μg/ml)	HU_{50}/μg of carbohydrate[a]	HU_{50}/μg of protein[a]
Filtered supernatant[b]	121	328	4.5	0.4	26.9
Postcentrifugation (CH)[c]	4,380	1,200	64	3.7 (9.3)	68.6 (2.6)
Sephacryl S-1000[d]	351	36.7	1.1	9.6 (24.0)	319.1 (11.9)
ACB–0.3 M NaCl[e]	210	43.2	2.4	4.9 (12.3)	87.5 (3.3)
ACB–0.4 M NaCl[f]	99	6.9	0.1	14.3 (35.8)	990.0 (36.8)

[a] Numbers in parentheses represent relative specific activity (HU_{50} per microgram of protein or HU_{50} per microgram of carbohydrate) compared with filtered supernatant.
[b] Supernatant from an exponential-phase culture of *E. coli* H-79 was filtered through a 0.45-μm-pore-size membrane filter.
[c] The filtrate was concentrated 43.7-fold and centrifuged at 235,000 × *g*.
[d] 7.5 ml of CH was fractionated by gel filtration with Sephacryl S-1000. Fractions with peak hemolytic activity were pooled for data shown (volume = 44 ml).
[e] 41 ml of pooled fractions from Sephacryl S-1000 column was fractionated by ion-exchange chromatography. AH eluted with ACB–0.3 M NaCl.
[f] AH eluted from an ion exchange column with ACB–0.4 M NaCl.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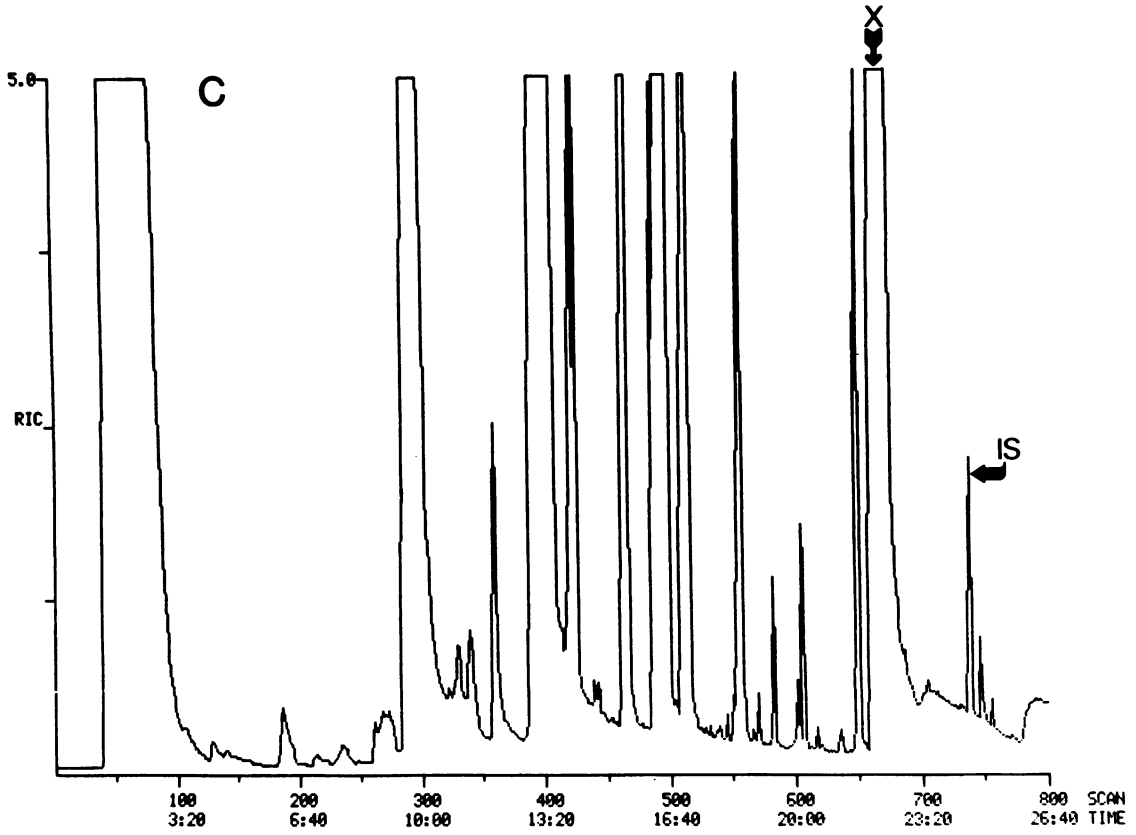

FIG. 3. Gas chromatograms of lipid extracts from 20 ml of ion-exchange-purified AH and LPS preparations. (A) ACB–0.3 M NaCl eluate (5,600 HU_{50}); (B) ACB–0.4 M NaCl eluate (2,640 HU_{50}); (C) 10 mg of *E. coli* H-79 LPS. Each sample was subjected to mild acid hydrolysis, saponified, and methylated. Extracts were concentrated to 20 μl and spiked with 2 μg of internal standard (IS). One microliter of sample was injected under conditions described in the text. A 3-hydroxytetradecanoic acid methyl ester peak (×) was identified in each sample by its mass spectrum.

Md.) directed against rabbit immunoglobulins or mouse immunoglobulin G and immunoglobulin M (heavy and light chains), respectively. Binding of the conjugated antibody was detected with a *p*-nitrophenyl phosphate substrate (Sigma). Monoclonal antibody subclass determination was done by a similar assay, using a panel of alkaline phosphatase-conjugated goat antisera specific for individual mouse antibody subclasses.

Hemolysin neutralization assay. Hybridoma culture supernatants (100 μl) were transferred to 96-well round-bottom polystyrene microtiter plates (Corning Glass Works). An equal volume of CH containing 25 HU_{50} per ml in saline with 10 mM $CaCl_2$ was mixed with each supernatant and incubated at 37°C for 30 min. A sheep erythrocyte suspension (2.5%) in saline (50 μl) was added to each well. The plates were incubated for 1 h at 37°C and observed visually for partial or complete inhibition of hemolysis.

Immunoblotting. Electrophoresed CH proteins in polyacrylamide gels were transferred to 0.2-μm-pore-size nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.). Electrophoretic transfer was accomplished overnight at 4°C with 200 mA (anodal migration) in 0.025 M Tris buffer (pH 8.3) containing 0.19 M glycine and 20% methanol, using a model TE52 Transphor unit (Hoefer Scientific Instruments, San Francisco, Calif.). Residual protein-binding sites on the membrane were blocked by incubation in 0.02 M Tris-buffered saline (pH 7.5) (TBS) containing 5% BSA (TBS-BSA) for 60 min at room temperature. The blocked nitrocellulose was transferred to an antibody probe solution consisting of either mouse ascites or rabbit antiserum, each diluted 1:50 in TBS-BSA. The membrane was incubated in the probe for 90 min and then washed four times (10 min each) in a bath of TBS. Reactivity of antibody probes with CH proteins was detected by incubating the nitrocellulose in horseradish peroxidase-conjugated goat antisera (Southern Biotechnology Associates, Birmingham, Ala.) with specificity for either rabbit immunoglobulins or mouse immunoglobulin G. After incubation and washing as above the nitrocellulose was transferred to a peroxidase substrate solution containing 60 mg of 4-chloro-1-naphthol (Sigma), 60 μl of 30% H_2O_2 (Fisher Scientific Co., Pittsburgh, Pa.), 20 ml of methanol, and 100 ml of TBS for detection of horseradish peroxidase-conjugated antibodies on the membrane.

Fatty acid detection. AH purified by ion-exchange chromatography was hydrolyzed by boiling for 4 h in 1% acetic acid to dissociate lipid A from LPS (9). The hydrolyzed samples (15 ml) were extracted overnight with 56 ml of chloroform-methanol (1:2, vol/vol). Phase separation was promoted by the addition of 20 ml of chloroform and centrifugation at 1,270 × *g*. The aqueous layer was removed and extracted again with 15 ml of chloroform. Both chloroform extracts were combined, washed with water, and concentrated to 2 ml at 40°C under vacuum in a Rotavapor R (Buchii, Switzerland) and then to a minimal volume under a stream of N_2. The concentrates were saponified with 0.5 ml of 7 N NaOH–2 ml of methanol (100°C, 60 min). The pH of the

samples was reduced with 0.5 ml of 5 N HCl, and each saponified sample was extracted twice with 2 ml of petroleum ether. The combined extracts were evaporated to a minimal volume under N_2 and methylated by incubation in 2 ml of methanol–0.5 ml of 6 N HCl (50°C, 30 min). The extracts were pooled, supplemented with docosanoic acid methyl ester (PolyScience Corp., Niles, Ill.) as an internal standard, evaporated to 20 μl under N_2, and stored at −20°C until analysis.

Lipids in each extract were separated with a model 9610 gas-liquid chromatograph (Finnigan MAT, San Jose, Calif.) equipped with a 30-m (0.25-mm internal diameter) SP-2330 silica capillary column (Supelco, Inc., Bellefonte, Pa.). The chromatograph was interfaced with a Finnigan model 4021 mass spectrometer for compound identification. A 1-μl sample was injected and held at 100°C for 3 min. The column temperature was increased to 280°C at a rate of 5°C/min. Chromatograms and mass spectra were recorded and interpreted by an Incos 2000 Data Acquisition/Processing System with access to the Environmental Protection Agency-National Institutes of Health reference library for compound search.

RESULTS

Purification of AH. As reported previously, hemolytic activity in the culture supernatants increased during exponential growth but decreased to undetectable levels during the stationary phase (24). Culture supernatants for AH purification were obtained during the late exponential phase when the hemolytic activity was maximal. AH in the filtered supernatant did not pass through XM-300 membranes during ultrafiltration, suggesting a molecular size in excess of 300,000 daltons. CH was further purified by a technique involving gel filtration and ion-exchange chromatography. Each of these chromatographic techniques recovered approximately 80% of the hemolytic activity applied to the column.

Initial attempts at purification of AH with Sephacryl S-300 (protein exclusion limit, 1.5×10^6 daltons) resulted in elution of hemolytic activity in the void volume (data not shown). With Sephacryl S-1000 (particle size exclusion limit, 300 to 400 nm) elution of hemolytic activity was retarded (see Fig. 1). AH elution correlated with elution of a broad protein peak and a shoulder on the carbohydrate elution profile. Peak hemolytic activity eluted at a volume of 284.5 ml. The k_{av} for this elution volume was 0.35. Nonhemolytic voided contaminants were well separated from AH by this technique.

Hemolytic activity recovered from Sephacryl S-1000 columns bound to DEAE-Sephadex at pH 4.0 (ACB–0.2 M NaCl). Figure 2 shows that AH eluted from the gel with the sequential addition of ACB–0.3 M NaCl and ACB–0.4 M NaCl. A similar amount of hemolytic activity was eluted with ACB–0.3 M NaCl and ACB–0.4 M NaCl. The fractions that eluted with ACB–0.4 M NaCl contained less protein and therefore had a higher specific activity (see Table 1).

Table 1 shows a summary of results from a typical purification run. Significant increases in specific activity of AH were achieved during CH preparation and by gel filtration with Sephacryl S-1000. Elution from ion-exchange columns with ACB–0.4 M NaCl and ACB–0.3 M NaCl separated AH into high- and low-specific-activity fractions, respectively. Carbohydrate was present in purified AH and copurified with protein in a reproducible manner.

LPS quantitation. Several lines of evidence suggested that the AH forms complexes with other macromolecules.

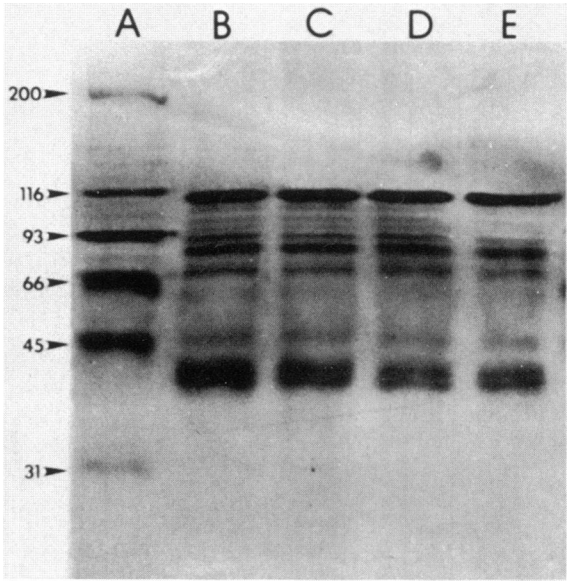

FIG. 4. SDS-PAGE profiles of hemolysin preparations. Ten to thirty microliters each of concentrated CH or AH from various stages of purification was mixed with sample buffer, boiled, and electrophoresed. Lanes: A, protein standards with molecular sizes shown in kilodaltons; B, CH; C, gel-filtration-purified AH; D, ion-exchange-purified AH (ACB–0.3 M NaCl eluate); E, ion-exchange-purified AH (ACB–0.4 M NaCl eluate). Each sample volume was adjusted to contain approximately 1,000 HU_{50}.

Among these are the large size and presence of carbohydrate in purified AH. These findings prompted us to search for LPS. Samples of AH eluted from DEAE-Sephadex columns with ACB–0.3 M NaCl and ACB–0.4 M NaCl were analyzed for 3-hydroxytetradecanoic acid as an indicator of LPS. Purified LPS from *E. coli* H-79 was analyzed for comparison. A major fatty acid (peak X in Fig. 3) with a retention time of 22 min was detected in all three samples. The mass spectrum of this methylated fatty acid was matched by computer library search with that of 3-hydroxytetradecanoic acid methyl ester (data not shown). By comparing the area of peak X in Fig. 3C (*E. coli* H-79 LPS) with that of the internal standard (IS), purified LPS was found to contain 2.2% 3-hydroxytetradecanoic acid (dry weight). Using this value, the concentrations of LPS in AH eluted from the ion-exchange column with ACB–0.3 M NaCl (280 HU_{50} per ml) and ACB–0.4 M NaCl (132 HU_{50} per ml) were calculated to be 18.6 and 0.65 μg/ml, respectively.

Analysis of proteins by SDS-PAGE. Concentrated AH preparations from each stage of purification were boiled in SDS–2-mercaptoethanol and electrophoresed in polyacrylamide gels. The electrophoretic protein profiles of the preparations shown in Fig. 4 were very similar. The major protein detected by Coomassie blue staining had a molecular size of 107,000 daltons. At least nine smaller proteins were also detected.

Characterization of monoclonal antibodies. Three hybridomas were isolated which reacted with CH but not with LPS in direct ELISA assays. One of these clones, D12E4E12, neutralized hemolytic activity. D12E4E12 and the two non-neutralizing CH antibodies (G11B7H4 and A10A3D7) belong to the immunoglobulin G2a subclass.

Identification of AH protein. Monoclonal antibodies were used as probes to detect AH proteins on nitrocellulose. The

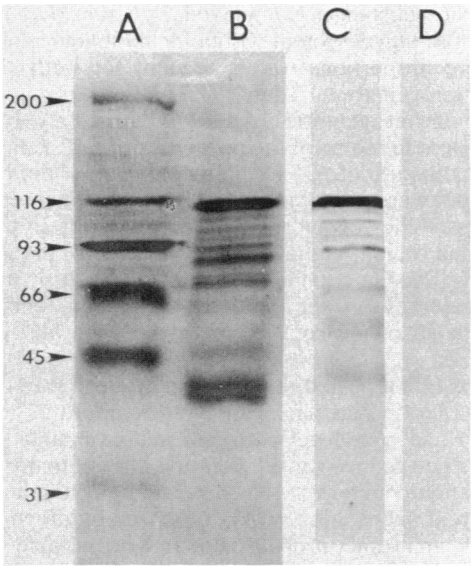

FIG. 5. Reactivity of CH proteins with D12E4E12 and control ascites. Lanes: A and B, Coomassie blue-stained SDS-PAGE profiles (A, protein standards with molecular sizes expressed as kilodaltons; B, CH [1,000 HU_{50}]); C and D, immunoblots of CH proteins on nitrocellulose (C, D12E4E12 ascites probe; D, X63-Ag8.653 control ascites probe).

107,000-dalton protein and numerous smaller minor proteins in CH reacted with D12E4E12 antibody (Fig. 5). The extracellular concentration of proteins increased during the exponential growth phase (7 to 11 h) of *E. coli* H-79 (Fig. 6A). This increase in proteins occurred concomitantly with the increase in hemolytic titer of the culture supernatants. During the stationary phase, when the hemolytic titer fell to undetectable levels, the electrophoretic profile of extracellular proteins was the same as that observed during logarithmic growth. Similar reactivity with D12E4E12 was observed for *E. coli* H-79 extracellular proteins collected at various stages of culture growth (Fig. 6B). No change in reactivity of any of the proteins occurred during the stationary growth phase when the hemolytic titer fell to undetectable levels. The nonneutralizing monoclonal antibodies (A10A3D7 and G11B7H4) also reacted strongly with the 107,000-dalton protein and numerous minor proteins (Fig. 7).

Immunoblotting with rabbit antisera. Immunoblotting with rabbit antisera was done to confirm that the reactivity of monoclonal antibodies with multiple CH proteins was not due to nonspecificity and that the proteins contained the same antigenic epitope. Protein bands excised from SDS-PAGE gels were used to prepare antiserum in rabbits. Rabbits were immunized with either the 107,000-dalton protein or with a combination of proteins with molecular sizes of less than 107,000 daltons. In either case, antisera obtained from the rabbits and used as probes reacted with several CH proteins including the 107,000-dalton protein (Fig. 8).

DISCUSSION

This study demonstrated several structural characteristics of *E. coli* AH and suggests that it is a protein complexed with other macromolecules such as LPS. We suspected that AH was very large because of its retention by XM-300 membranes and elution from Sephacryl S-300 columns in the void volume. A large size is supported by other published data (3, 13, 22). The only gel matrix that retarded the flow of AH during gel filtration was Sephacryl S-1000. According to the manufacturer, particles with diameters greater than 300 to 400 nm are voided by this gel. The 60-nm bovine papillomavirus elutes on Sephacryl S-1000 with a k_{av} of 0.5 (21). Therefore, the particle size for AH (k_{av} = 0.35) is greater than 60 nm. However, the broad elution peak observed for AH suggests a size heterogeneity.

Felmlee et al. (8) recently reported that the pI of the *hlyA* protein based on its predicted amino acid sequence is 6.1. The binding of AH to DEAE-Sephadex at pH 4.0 and its salt elution characteristics suggest that the nondenatured hemolysin is anionic and heterogeneous. In addition, isoelectric focusing suggests that the hemolysin has a pI of less than 3.6 (unpublished data). The lower pI observed in this study could be due to charge contributions from other

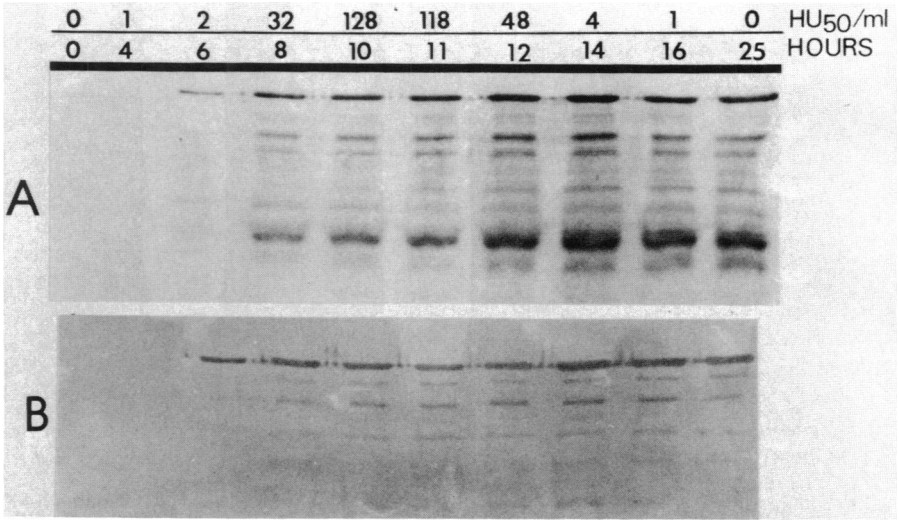

FIG. 6. SDS-PAGE and immunoblots of *E. coli* H-79 supernatant proteins from various stages of growth. Samples (10 ml) of filtered culture supernatants harvested at various timepoints (time of harvest and hemolytic titers shown) were concentrated 200-fold and analyzed by (A) SDS-PAGE and (B) immunoblotting identical preparations transferred to nitrocellulose and probed with D12E4E12 ascites.

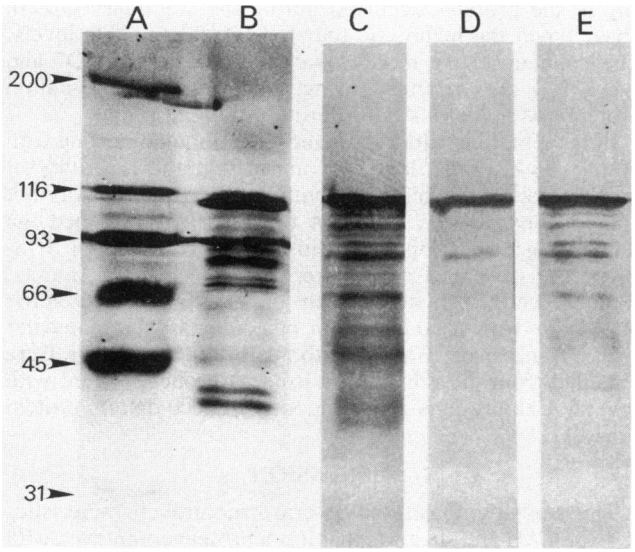

FIG. 7. Comparison of reactivity of CH proteins with hemolysis-neutralizing and nonneutralizing monoclonal antibodies. Lanes: A and B, Coomassie blue-stained SDS-PAGE profiles (A, protein standards with molecular sizes expressed as kilodaltons; B, CH [1,000 HU_{50}]); C through E, immunoblots of CH proteins on nitrocellulose (C, A10A3D7 ascites probe; D, G11B7H4 ascites probe; E, D12E4E12 ascites probe).

more acidic substances complexed with the *hlyA* protein. Binding of the *hlyA* protein with a highly anionic substance in different proportions would account for both size and charge heterogeneity.

The significant quantities of LPS in purified AH fractions could account for some of the properties of AH. A large size and anionic nature of AH would be expected if the hemolysin proteins were complexed with LPS. Felmlee et al. (8) detected stretches of hydrophobic amino acids in the *hlyA* protein that could potentially interact with the lipid A portion of LPS. We obtained inconsistent results in attempts to quantitate LPS by the limulus lysate assay. This inconsistency could result from interactions of the *hlyA* protein with lipid A.

Williams (27) reported the isolation of a cell-free hemolysin from *E. coli* cultures in an uncomplexed form. The active hemolysin had a molecular size of approximately 120,000 daltons as determined by gel filtration chromatography and sedimentation equilibrium. He suggested that the large size and apparent aggregation of AH observed by other investigators was an artifact probably due to hydrophobic interaction among *hlyA* proteins in pure solution. We do not believe this is the case for our AH preparations for several reasons. First, aggregates composed solely of the *hlyA* protein would not be expected to have the extremely low pI observed in our studies. Second, we observed identical k_{av} values with Sephacryl S-1000 columns for hemolysin preparations irrespective of their purity, concentration, or the medium used for their production (unpublished data). It is possible that some strain differences do occur and that AH complex formation requires certain cell properties not characteristic of all strains.

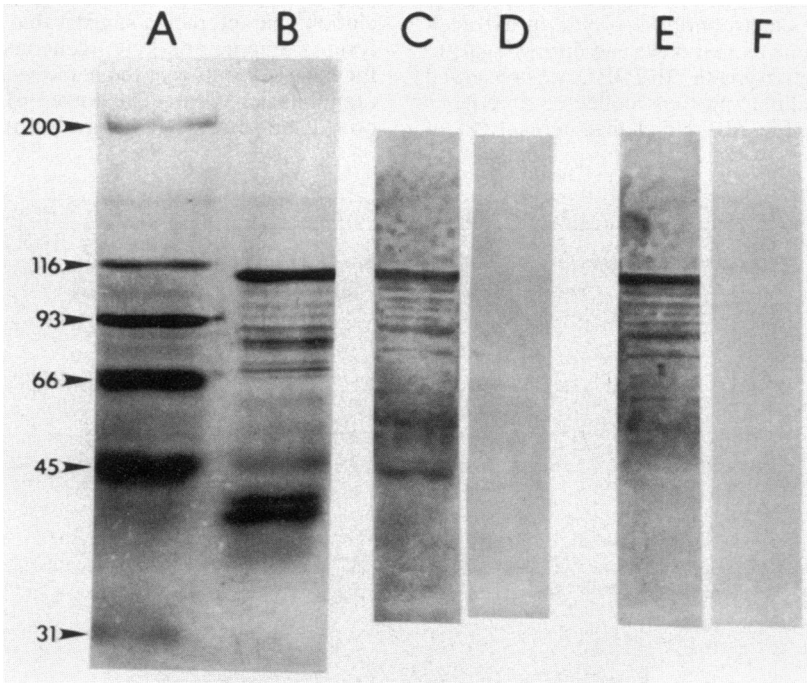

FIG. 8. Reactivity of CH proteins with polyclonal rabbit antisera. Lanes: A and B, Coomassie blue-stained SDS-PAGE profiles (A, protein standards with molecular sizes expressed as kilodaltons; B, CH [1,000 HU_{50}]); C through F, immunoblots of CH proteins on nitrocellulose. Nitrocellulose strips were probed with serum from a rabbit immunized with the 107,000-dalton CH protein (C, postimmunization; D, preimmune) or with serum from a rabbit immunized with the combined smaller (<107,000-dalton) CH proteins (E, postimmunization; F, preimmune).

Our demonstration of a 107,000-dalton protein in *E. coli* H-79 culture supernatants and purified AH preparations is in agreement with Felmlee et al. (8) and Mackman and Holland (18) who proposed that the 106,000- to 110,000-dalton *hlyA* protein is exported intact from the bacterial cell. No 58,000-dalton protein as reported by Noegel et al. (20) was detected. Further, we provided evidence by use of the hemolysin-neutralizing monoclonal antibody D12E4E12 that the 107,000-dalton protein detected in this study is the product of the *hlyA* gene.

In addition to the 107,000-dalton protein at least nine other smaller proteins were detected in CH and purified AH preparations. All of these reacted with D12E4E12 and were shown to be antigenically similar when used to immunize rabbits. Therefore, as suggested by other investigators (7), the smaller proteins appear to be proteolytic degradation products of the *hylA* protein. Two other anti-AH monoclonal antibodies that reacted with the 107,000-dalton protein also reacted with the smaller proteins. The proteins in SDS-PAGE profiles of AH preparations were nearly identical, qualitatively and quantitatively, irregardless of the stage of purification or specific activity. These findings have several implications. First, results of immunoblots suggested that *E. coli* H-79 exports very few if any extracellular proteins that are retained by XM-300 membranes other than hemolysin proteins. Immunoblotting results also suggested that proteolytic degradation occurs at a site on the protein not recognized by the hemolysin-neutralizing monoclonal antibody. Finally, proteolysis may result in the generation of small peptides which migrate off the SDS-PAGE gels and are undetectable. This could explain why proteins detected in AH preparations with different specific activities were quantitatively similar after electrophoresis of the same number of hemolytic units.

The 107,000-dalton protein and its proteolytic peptides contain an antigenic epitope required for hemolytic activity. However, it is impossible to say which of these are hemolytically active since the denaturing conditions required for their separation inactivate the hemolysin. Proteolysis does not appear to occur extracellulary since the amount of the 107,000-dalton protein did not decrease nor did the amounts of the smaller proteins increase in relative proportions during culture incubation. This also implies that proteolysis is not responsible for loss of hemolytic activity in the supernatant during the stationary phase.

The mechanism of release of the *hlyA* protein from the outer membrane is incompletely understood. Hemolysin exists transiently in a hemolytically active form on the external surface of the outer membrane (25). Final release from the cell is dependent upon *hlyD* (*hlyB*$_b$) (8, 25), a gene whose specific function is unknown. Our findings are consistent with several possible release mechanisms. The hemolysin may be exported as a single molecule that complexes with other components from the bacterial cell that are released into the medium. Another possibility is that the product of the *hlyA* gene is an integral protein in the outer membrane. The configuration required for hemolytic activity may or may not be dependent upon its association with the membrane lipids. The function of *hlyB*$_b$ (*hlyD*) may be to direct selective fragmentation or blebbing (17) of the outer membrane in areas that contain the *hlyA* protein. In such cases, AH would be released as a complex with outer membrane materials. Consistent with this model are results reported by others that hemolytic activity is destroyed by lipase treatment (4). Phenotypic characteristics of *E. coli* cells other than those determined by the *hly* genes are reported to influence hemolysin production and release. For instance, Juarez and Goebel (14) produced chromosomal mutations in *E. coli* cells carrying a recombinant *hly* plasmid that appeared to affect the cell envelope. The recombinants produced small zones of hemolysis typical of mutants that produce cell-bound extracellular hemolysin but cannot release it into the medium. One can speculate that the defect in these mutants alters the outer membrane so that recognition of hemolysin-containing regions in the membrane and their selective fragmentation does not occur.

Further characterization of AH complexes is required before their significance can be assessed. We are currently preparing immunoadsorbents with anti-AH monoclonal antibodies to determine the chemical composition of affinity-purified hemolysin. A close association of LPS or other cellular components with AH could have significant consequences affecting the biological properties and toxicity of hemolytic *E. coli* strains. Also, this association could indicate a unique mechanism for release of AH from the cell.

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