THE MEMBRANE ATTACK MECHANISM OF COMPLEMENT: THE THREE POLYPEPTIDE CHAIN STRUCTURE OF THE EIGHTH COMPONENT (C8)*‡

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We wish to report that the eighth component of human complement (C8), which has an unusual function, has an unusual subunit structure. This paper presents information on the polypeptide chain structure of the molecule, the different modes by which the chains are linked to each other, and the differential availability of the chains for radiolabeling in the intact C8 molecule. This information permits the design of a tentative model of the C8 molecule that pertains to its function in cell lysis. The work was afforded by the elaboration of a modified method of isolation which allows the preparation of milligram amounts of the protein.

C8 plays a central role in the causation of membrane damage by the C5b-9 complex (1–4). The million dalton complex is assembled upon activation of C5 by the C5 convertase of either the classical or the alternative complement pathway (5–7). Examination of the sequential steps which lead to membrane bound C5b-9 has shown that attachment of the C5b,6,7 trimolecular complex has no untoward effects on membrane function (1–5). However, binding of C8 to this complex results in membrane damage. C8-induced cytolysis is proportional to the number of C5b-8 complexes bound per cell[1] and greatly accelerated by binding of C9 (1, 2, 4). Accordingly, the C8 molecule is considered to be directly responsible for the production of complement-dependent membrane injury.

Materials and Methods

Serum and Complement Components. Serum was obtained from outdated human plasma by addition of $CaCl_2$ to a final concentration of 20 mM. Clotting proceeded at 37°C for 2 to 3 h, and the serum was stored overnight at 4°C. The serum was obtained by wringing the clot in nylon gauze. C9 was prepared as previously described (8).

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[1] Kolb, W. P., J. A. Haxby, and H. J. Müller-Eberhard. The mechanism of action of the eighth component of human complement. Manuscript in preparation.

C8 Hemolytic Assay. Samples to be assayed for C8 activity were incubated with 3×10^7 EAC1-7 (5) and an excess of C9 (100 $CH_{50}U$) at 37°C in a final volume of 500 μl Veronal-buffered saline, pH 7.4, containing 1.5×10^{-4} M $CaCl_2$, 5×10^{-4} M $MgCl_2$ and 0.1% gelatin (GVB).[2] The reaction was terminated by transferring the tubes to an ice bath, addition of 1 ml ice cold GVB, and immediate centrifugation. Release of hemoglobin into the supernates was quantitated spectrophotometrically at 412 nm.

Isolation Procedure for C8

AMMONIUM SULFATE PRECIPITATION. All steps were carried out at 4°C. 1 liter of serum was diluted with 400 ml of distilled water and adjusted to pH 6.9 with 0.1 N HCl. Solid ammonium sulfate (294 g) Mallinckrodt Inc., St. Louis, Mo.) was added with constant stirring to achieve a saturation of 37.5%. After 1 h, the precipitate was collected by centrifugation at 5,000*g* for 15 min and utilized for the preparation of C7. Solid ammonium sulfate (104 g) was added to the supernate to achieve a saturation of 50% and the precipitate collected as described above.

CM-32 CELLULOSE CHROMATOGRAPHY. The ammonium sulfate precipitate (50% saturation) was resuspended in 600 ml of CM-32 starting buffer (phosphate buffer pH 6.0, conductance 5.5 mmho/cm) and dialyzed for 16 h against 2 × 10 liters of this same buffer. The sample was applied to a 7.5 × 50-cm CM-32 column equilibrated with 5 liters of the starting buffer. The C8 was eluted by a linear salt gradient made with 3 liters starting buffer and 3 liters starting buffer adjusted to a conductance of 35 mmho/cm with NaCl.

QAE-Sephadex Chromatography. The C8-containing fractions from the CM-32 column were pooled and dialyzed against 10 liter of the QAE starting buffer (phosphate buffer pH 8.0, conductance 6.0 mmho/cm) for 16–36 h. The sample was applied to a 3.5 × 60-cm QAE-Sephadex column equilibrated with the starting buffer. The column was washed with 3 liter of starting buffer, and C8 was then eluted with a linear salt gradient using 2 liter starting buffer and 2 liter starting buffer adjusted to a conductance of 30 mmho/cm with NaCl. C8-containing fractions were pooled and concentrated by positive pressure ultrafiltration employing an Amicon UM-10 membrane (Amicon Corp., Lexington, Mass.). The purified C8 was passed through a 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass.), divided into aliquots, frozen in liquid nitrogen, and stored at −70°C.

Radioiodination. Highly purified C8 and marker proteins were trace radiolabeled with either ^{125}I or ^{131}I by the method McConahey and Dixon (9). C8 was radiolabeled in either phosphate-buffered saline or in the presence of 1% sodium dodecyl sulfate (SDS) and 4–8 M urea. Fully reduced C8 was radioiodinated by incubation in 1% SDS, 5 M urea, and 1% 2-mercaptoethanol (vol/vol) at 37°C for 16 h. Before iodination this solution was dialyzed for 16 h at 22°C against 2 × 2 liters of 0.05 M phosphate buffer, pH 7.4, containing 1% SDS to remove the 2-mercaptoethanol which would inhibit the iodination reaction. Radiolabeling of C8 in the presence of SDS was conducted employing 500 μg chloramine T and 5 mCi of isotope per mg of protein. C8 in its native state was radiolabeled employing 10–25 μg chloramine T and 1–5 mCi of isotope per mg of protein.

SDS Polyacrylamide Gel Electrophoresis. Electrophoresis was conducted using the continuous phosphate-buffered system of Weber and Osborn (10). 5–9% gels were made with 0.09 M phosphate buffer, pH 7.4, and 2% SDS. The upper and lower electrode vessels contained a 1:2 dilution of this buffer. Samples were prepared by incubation at 37°C for 16 h with 5 M urea, 1% SDS, and 1% 2-mercaptoethanol (if reduction was intended). For molecular weight determinations of C8 subunits, radioiodinated HgG, BSA, C6, ovalbumin, chymotrypsinogen and cytochrome C were employed as internal markers in various combinations. 10–20 μl of glycerol and 10 μl of 2% bromophenol blue were added to each sample before electrophoresis, which was conducted at a constant current of 8 mA/gel until the tracking dye had traveled to within 1 cm of the bottom of the gel. Analytical gels were 6 × 120 mm and were fixed and stained for protein simultaneously in methanol, acetic acid, and water (10:1:9) containing 0.01% Coomassie Brilliant Blue (K & K Laboratories, Inc., Plainview, N. Y.). Gels were destained electrophoretically in 7.5% acetic acid, the stained protein bands marked with copper wire, the gels sliced in 2-mm segments, and ^{125}I and ^{131}I radioactivity determined in a refrigerated Packard autogamma scintillation spectrometer Model 5260 (Packard Instrument Co. Inc., Downers Grove, Ill.).

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[2] *Abbreviations used in this paper:* GVB, Veronal-buffered saline containing 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatin; PAS, periodic acid Schiff; SDS, sodium dodecyl sulfate.

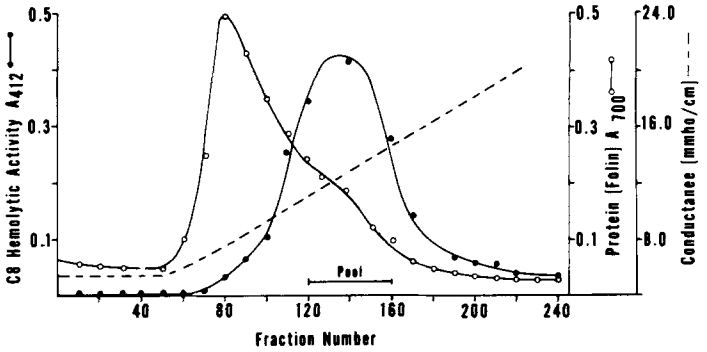

FIG. 1. CM-32 column chromatography as the second step in the purification of human C8. The ammonium sulfate precipitate, 37.5–50% saturation, was resuspended in and dialyzed against phosphate buffer pH 6.0, conductance 5.5 mmho/cm. The sample was applied to a 7.5 × 50-cm CM-32 cellulose column equilibrated with the same buffer. A major portion of the protein was eluted by washing the column with starting buffer for 16–24 h at a flow rate of 250 ml/h before application of a linear salt gradient. C8 containing fractions were pooled as indicated.

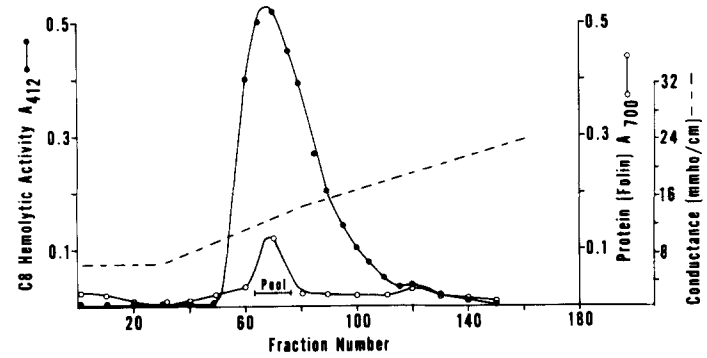

FIG. 2. QAE-Sephadex column chromatography as the third step in the purification of human C8. The CM-32 pool was dialyzed against and applied to a 3.5 × 60-cm QAE-Sephadex column equilibrated with phosphate buffer pH 8.0, conductance 6.0 mmho/cm. The column was washed with 3 liters of this buffer at a flow rate of 150 ml/h before application of a linear salt gradient. The C8 containing fractions were pooled as indicated and concentrated to 3 ml.

The carbohydrate content of the C8 subunits was visualized and quantitatively estimated by the periodic acid Schiff (PAS) staining procedure as outlined by Glossman and Neville (11). PAS-stained gels were scanned at 550 nm employing a Gilford model 200 recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and a Beckman DU spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.).

Molar Ratios. Estimation of the molar ratios of the C8 subunits was conducted as previously described for the subunits of the C5b-9 complex (7). Briefly, gels stained with Coomassie Blue were spectrophotometrically scanned at 600 nm, and the area of each peak recorded on graph paper was determined on the basis of its weight. Relative moles of each protein were calculated by dividing the weight of each area by the molecular weight of the corresponding subunit. Molar ratios for both reduced and nonreduced C8 were expressed relative to C8 β.

Results

Modified Method of Isolation of C8. Ammonium sulfate fractionation of whole human serum showed that up to 90% of C8 was precipitated from serum at

TABLE I
Isolation of C8 from Human Serum

Step	Procedure
1. Ammonium sulfate fractionation	37.5-50% of saturation
2. CM-32 cellulose cation exchange chromatography	pH 6.0, linear NaCl gradient 5.5-35 mmho/cm
3. QAE-Sephadex anion exchange chromatography	pH 8.0, linear NaCl gradient 6.0-30 mmho/cm

TABLE II
*Purification of C8**

Fraction	Volume	Protein		Activity‡ $\times 10^{-12}$	Activity		Yield		Purification
		Conc	Total		Specific $\times 10^{-10}$	Total em $\times 10^{-13}$	Activity	Protein	
	ml	*mg/ml*	*mg*	*em/ml*§	*em/mg*		%	%	
Outdated whole human serum	2,000	64.5	129,000	0.43	0.66	85.72	100	100	1
Ammonium sulfate precipitate 37.5-50%	500	31.0	15,500	1.54	4.95	76.92	89.7	12.0	7.5
CM-32	30	1.85	55.5	4.09	221.10	12.27	14.3	0.043	333
QAE-Sephadex	2	2.68	5.36	33.33	1,243.78	6.66	7.8	0.004	1,883

* Values derived from C8 preparation no. 70.
‡ Activity assayed with EAC1-7 plus excess C9 for 30 min at 37°C.
§ em, effective molecules.

37.5-50% saturation. Chromatography of the dissolved ammonium sulfate precipitate on CM-32 cation exchange cellulose eliminated 99.5% of the applied protein. As shown in Fig. 1, C8 was eluted at a conductance of 12.4 mmho/cm with a linear NaCl gradient and was found to be virtually free of all other terminal complement components. Final separation of C8 from the contaminating proteins was achieved by anion exchange chromatography employing QAE-Sephadex. The C8 protein and hemolytic activity are eluted at a conductance of 12 mmho/cm by a linear NaCl gradient. As shown in Fig. 2 the distribution of protein and activity correlated well. The three-step isolation procedure is summarized in Table I. The yield of C8 activity varied between 1.8 and 9% of the activity present in serum. The purification was 1,200-2,000-fold (Table II).

C8 Subunit Structure. The subunit structure of C8 was examined by SDS polyacrylamide gel electrophoresis with and without 2-mercaptoethanol. Fig. 3, upper panel, depicts the pattern of SDS-urea-dissociated C8 and the corresponding spectrophotometric analysis of the gel. The lower panel shows the pattern of C8 dissociated by SDS and urea in the presence of 1% 2-mercaptoethanol. These gel patterns are representative for 16 different C8 preparations and 150 separate analyses and clearly demonstrate the three polypeptide chain structure of C8. The molecular weights of the fully reduced C8 subunits, as listed in Table III, were determined to be: C8α, 77,000; C8β, 63,000; and C8γ, 13,700 and of the

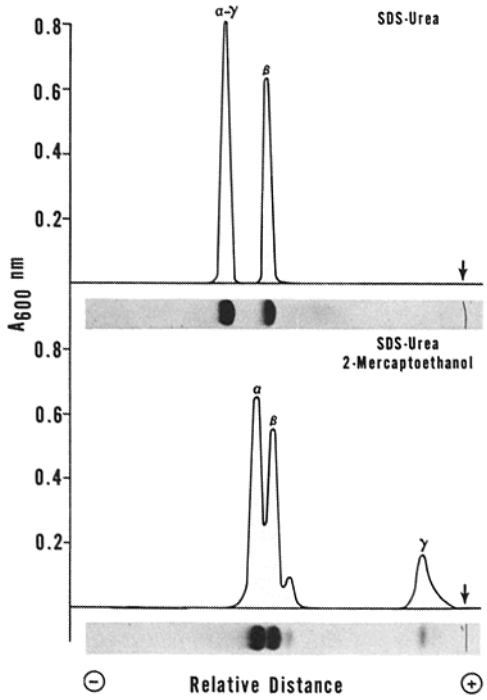

FIG. 3. Demonstration of C8 subunit structure by SDS polyacrylamide gel electrophoresis. Purified C8 was treated with 1.0% SDS and 4 M urea in the presence (bottom panel) or absence (top panel) of 1% 2-mercaptoethanol before electrophoretic separation in a 7.5% gel at 8 mA/gel. The gels were stained with 0.02% Coomassie Blue and, subsequent to destaining, were spectrophotometrically scanned at 600 nm.

nonreduced subunits: C8α-γ, 99,000 and C8β, 75,000. Staining of reduced C8 gels for carbohydrate with PAS revealed equal but moderate staining of C8α and C8β with no staining of C8γ.

Molar ratios of C8 subunits were estimated from the area under the peaks obtained from the spectrophotometric tracings as seen in Fig. 3. These results, presented in Table IV, indicate that the C8 subunits occur in equimolar amounts.

Differential Iodination of C8 Subunits. Fig. 4 demonstrates that the β- and γ-chain of native C8 take up isotope upon iodination by the chloramine T method, while the α-chain remains totally unlabeled. Essentially all the radioactivity present the α-γ subunit is found in the γ-chain after reduction. However, that the tyrosine residues of the α-chain can be labeled to the extent theoretically expected was shown by iodination in the presence of the denaturing agents SDS and urea (Fig. 4).

Discussion

The development of an isolation procedure for obtaining milligram quantities of highly purified human C8 is a prerequisite for in depth structural studies of the molecule which are required for an understanding of C8's key role in the causation of immune cytolysis. The described C8 purification procedure yielded

TABLE III
Subunit Composition of C8 as Analyzed by SDS-Polyacrylamide Gel Electrophoresis

C8 subunit	Dissociating conditions	Observed molecular weight*
α-γ	1% SDS, 5 M urea	99,000
β	1% SDS, 5 M urea	75,000
α	1% SDS, 5 M urea, 1% 2-mercaptoethanol	77,000
β	1% SDS, 5 M urea, 1% 2-mercaptoethanol	63,000
γ	1% SDS, 5 M urea, 1% 2-mercaptoethanol	13,700

* Molecular weight values were determined by coelectrophoresis with radiolabeled internal protein markers.

TABLE IV
Molar Ratios of C8 Subunits as Determined by Scanning of SDS Polyacrylamide Gels Stained with Coomassie Blue

C8 subunit	Reduction	Stain intensity*	Moles	Molar ratio‡
		A	$A/mol\ wt \times 10^{-5}$	
α-γ	–	5.72	5.78	0.94
β	–	4.57	6.09	1.0
α	1% 2-mercaptoethanol	2.80	3.64	0.81
β	1% 2-mercaptoethanol	2.83	4.49	1.0
γ	1% 2-mercaptoethanol	0.56	4.07	0.91

* The stain intensity (A_{600}) for each subunit was determined by the weight (g) of the graph paper corresponding to the area under each peak.
‡ Molar ratios for both reduced and nonreduced subunits are expressed relative to C8β.

1.8-9% of the C8 activity present in serum and afforded a 1,200-2,000-fold purification. This degree of purification was greater than theoretically expected (1,000-1,200-fold), considering a C8 concentration in serum of 80 μg/ml (12).

SDS polyacrylamide gel electrophoretic analysis of fully reduced C8 revealed three nonidentical polypeptide chains with the following molecular weights: C8α, 77,000; C8β, 63,000; and C8γ, 13,700. SDS-urea treatment dissociated C8 into two subunits, C8α-γ, 99,000 and C8β, 75,000. The molecular weight determinations for fully reduced proteins in the presence of SDS are more reliable than those determined for unreduced proteins. The high molecular weight values obtained for unreduced proteins are due to incomplete unfolding of the protein and therefore incomplete binding of SDS (13). The combined mol wt of the reduced α-, β-, and γ-chains is 153,700, which agrees well with the calculated molecular weight of 153,000 obtained from sedimentation and diffusion coefficients previously reported (14).

All C8 preparations examined to date contain a variable amount of two peptides that have mol wt, respectively, of 55,000 and 12,000 daltons. They probably represent fragments of C8β which are produced by limited proteolysis during purification and are held together by disulfide bonds. That these frag-

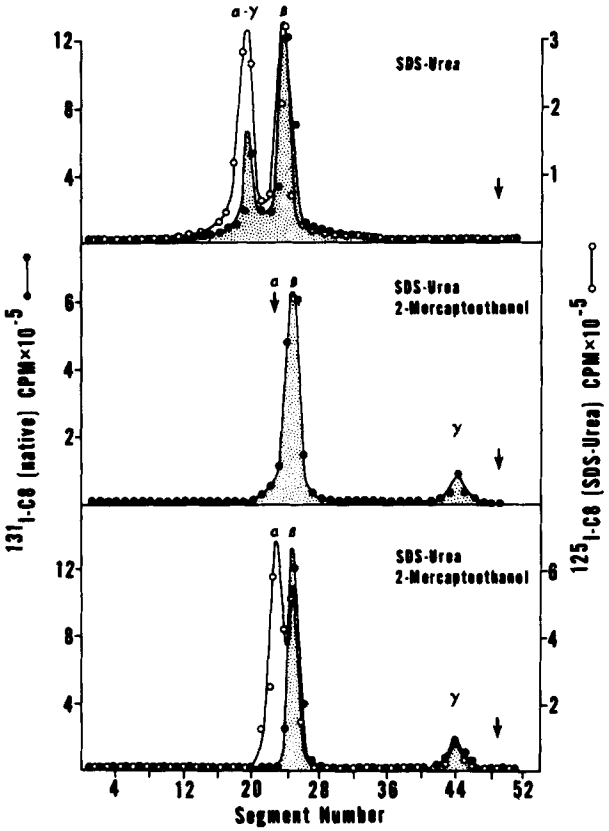

FIG. 4. Selective radioiodination of the β- and γ-chains in the native C8 molecule. 200 μg of purified C8 was labeled with ^{131}I in phosphate-buffered saline and 300 μg was labeled with ^{125}I in 0.2% SDS and 4 M urea by the chloramine T procedure. Before SDS polyacrylamide electrophoresis, portions of the labeled C8 samples were mixed with 50 μg of unlabeled C8 and the mixtures treated with SDS and urea with and without 2-mercaptoethanol. The protein bands, as visualized by staining, were marked with copper wire and the gels sliced into 2-mm segments and the distribution of ^{125}I and ^{131}I radioactivity was determined. (●—●), C8 labeled in native form; (○—○), C8 iodinated in the presence of SDS and urea. Top: sample treated with 0.2% SDS, 4 M urea; middle and bottom: sample treated with 0.2% SDS, 4 M urea, 1% 2-mercaptoethanol. The position of the stained protein bands is indicated by the Greek symbols of the subunits.

ments represent authentic subunits of C8 is precluded by their presence in amounts less than 0.2 mol per mol of protein.

The almost total lack of labeling of the α-chain upon radioiodination of native C8 is remarkable. Examination of 15 different preparations of radiolabeled C8 showed iodine substitution of the β- and γ-chain, but not of the α-chain. That the α-chain is able to take up iodine was demonstrated by iodination of denatured C8. We interpret these results to indicate that the α-chain relative to the two other chains is located primarily in the interior of the intact molecule. If this interpretation is correct, it would suggest that the α-chain is more hydrophobic than the other two chains. In that case, the α-chain may represent the mem-

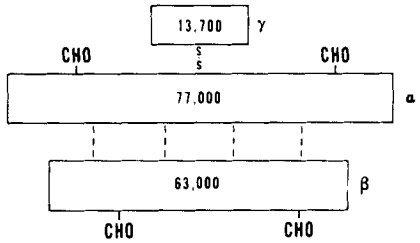

FIG. 5. Schematic representation of the chain structure of C8 indicating that the α- and γ-chains are covalently linked and that the β-chain is bound to the other two chains by noncovalent forces.

brane insertion unit of C8 and of the C5b-9 complex, which in the native C8 molecule is protected from contact with water by an outer shell provided by the β- and γ-chain. C1q, although a totally differently structured molecule, also exhibits differential availability of its chains to labeling with radioiodine (15).

Fig. 5 depicts a highly schematic representation of the chain structure of the C8 molecule. The basic features of the model are the following: The molecule is composed of two noncovalently linked subunits (α-γ and β) and of three nonidentical polypeptide chains. The largest (α) and the smallest (γ) chains are disulfide bonded. The α- and β-chains, but not the γ-chain, contain carbohydrate. The position of the chains indicated in Fig. 5 is arbitrary. However, it is proposed that the α-chain is located relatively in the interior, and the other two chains relatively on the surface of the native C8 molecule. Such an arrangement would result in a compact spherical conformation. Hydrodynamic measurements (F/F_0 = 1.2) and electron microscopic visualization[3] of C8 have indeed indicated an almost spherical shape of the molecule. The proposed topology of the chains implicitly suggests that the intact C8 molecule, as it occurs in the extracellular fluids, harbors a concealed membranophilic polypeptide chain which, upon attachment to membrane bound C5b-7, becomes instrumental in the formation of the initial complement-dependent transmembrane channel.

Summary

The purification of human C8 in milligram quantities from outdated human serum was achieved by ammonium sulfate precipitation (37.5–50% saturation) and ion exchange column chromatography employing CM-32 cellulose and QAE-Sephadex. The yield of C8 activity ranged from 2–9%, and the average purification was 1,700-fold.

Fully reduced C8 was shown by SDS polyacrylamide gel electrophoresis to have three polypeptide chains which were present in equimolor ratios: α, 77,000 daltons; β, 63,000 daltons; and γ, 13,700 daltons. C8 denaturation by SDS and urea in the absence of reducing agents revealed two noncovalently linked subunits: α-γ, 99,000 daltons, and β, 75,000 daltons.

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[3] Kolb, W. P., W. Krebs, E. Podack, and H. J. Müller-Eberhard. Ultrastructural and hydrodynamic properties of the soluble C5b-9 complex of human complement and its precursors. Manuscript in preparation.

When C8 was radioiodinated in native form by the chloramine T method, iodine was taken up by the β- and γ-chains, but not by the α-chain. However, all three chains were iodinated to the extent theoretically expected in the presence of SDS and urea. These observations suggest that the α-chain is located in the interior of the intact molecule, and that the β- and γ-chains are exposed on its surface. The probable relationship of the subunit topology to the cytolytic function of C8 is discussed.

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