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The Isolation and Structure of C4, the Fourth Component of Human Complement

IRMA GIGLI,* INGE VON ZABERN† and RODNEY R. PORTER

Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

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The fourth component of complement, C4, was isolated from human serum in good yield, and in confirmation of previous reports was shown to be formed from three peptide chains, α, β and γ, with apparent mol.wts. 90000, 80000 and 30000 respectively. Preparative methods are described for the isolation of the three peptide chains and their amino acid analyses reported. Component C4 contains 7.0% carbohydrate, α-chain 8.6% and the β-chain 5.6%. The *N*-terminal amino acid sequences are given for 12 residues of the α-chain, eight of the β-chain and 19 of the γ-chain.

Initiation of the activation of complement in blood by antibody–antigen complexes is principally through the classical pathway in which the first component, C1,‡ is activated, and this in turn converts components C2 and C4 into forms which interact, giving rise to the complex proteinase $\overline{\text{C4–C2}}$, also known as C3-convertase (reviewed by Reid & Porter, 1975). Little is known of the structure and activity of the $\overline{\text{C4–C2}}$ complex, and to investigate this a study has been made of the isolation and structure of component C4. Earlier work (Schreiber & Müller-Eberhard, 1974; Cooper, 1975; Nagasawa & Stroud, 1976) has shown that component C4 is a protein of 200000 mol.wt., with three peptide chains, α, β and γ, of apparent mol.wts. 93000, 78000 and 33000 respectively.

Electrophoretic heterogeneity, probably due to a structural polymorphism, has been reported in component C4 of human serum (Rosenfield *et al.*, 1969) and much interest has been aroused by the observation that component C4 of mouse serum may be identical with the Ss protein, a polymorphic serum protein which is linked genetically with the major histocompatibility complex (Meo *et al.*, 1975; see also Lachmann *et al.*, 1975; Curman *et al.*, 1975). Subsequently evidence has been given that a component-C4 structural gene maps in this complex (Teisberg *et al.*, 1976). As the activated component C4 has been shown to bind to cell surfaces (Budzko & Müller-Eberhard, 1970; Patrick *et al.*, 1970), this raises the possibility that it may also be concerned in the complex cellular interactions involved in an immune response.

In the present paper, simple methods are described for the preparation in good yield of component C4 and also of its three polypeptide chains. Many but not all of the previous reports on their structure have been confirmed and additional information has been obtained.

Materials and Methods

Materials

Chemicals were obtained as follows: iPr_2P-F, Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; mannitol and hexosamine hydrochlorides, BDH, Poole, Dorset, U.K.; neutral sugars and sialic acid standards, Sigma; prepacked columns for g.l.c., Phase-Sep, Queensferry, Clwyd, Wales, U.K.; sodium dodecyl sulphate, BDH; toluene-*p*-sulphonic acid, 3-(2-aminoethyl)indole hydrochloride and silylating reagents, Pierce and Warriner, Chester, U.K.; iodo[1-^{14}C]acetamide (60mCi/mmol), The Radiochemical Centre, Amersham, Bucks., U.K.; DEAE-Sephadex A-50, Sepharose 6B, CL-Sepharose 6B and Sephadex G-200, Pharmacia Fine Chemicals, Uppsala, Sweden; Dowex 1 (X8; 200–400 mesh) Sigma; hydroxyapatite (DNA grade), Bio-Gel HTP, Bio-Rad Laboratories, Bromley, Kent, U.K.

* Present address: New York University Medical Center, Skin and Cancer Unit, 562 First Avenue, New York, NY 10016, U.S.A.

† Present address: Max-Planck-Institut für Experimentelle Medizin, Abteilung Biochemische Pharmakologie, Herrmann-Rein-Strasse 3, 3400 Göttingen, Germany.

‡ Abbreviations: the nomenclature of complement components and subcomponents is that recommended by the World Health Organization (1968). Activated components are indicated by a bar, e.g. $\overline{\text{C4}}$. iPr_2P-F, di-isopropyl phosphorofluoridate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Outdated human plasma after cryoglobulin precipitation was obtained from the Churchill Hospital, Oxford. $CaCl_2$ was added to 20mM and the plasma left to clot overnight at 4°C. The clot was centrifuged down (2000g, 30min) and the serum stored at −20°C.

Haemolytic assay of component C4

Gelatin/veronal buffers were prepared as described by Nelson *et al.* (1966). Dilutions (0.5ml) of component C4 were incubated at 30°C with 0.5ml of erythrocytes (10^8/ml) sensitized with antibody and C1, the first component of complement. After addition of component C2, the haemolytic reaction was completed by addition of 1ml of guinea-pig serum diluted 1:15 (v/v) in 0.04M-EDTA in gelatin/veronal buffer; then 4.5ml of cold 0.15M-NaCl was added after incubation for 1h at 37°C and the mixture was centrifuged. The degree of lysis was measured by reading the A_{410} of the supernatant. Results were expressed in 50%-lysis (H50) units multiplied by the number of erythrocytes per ml (10^8) and referred to the titre of a standard serum stored at −70°C.

Electrophoresis in polyacrylamide gel

This was performed with buffers containing sodium dodecyl sulphate, as described previously (Gigli *et al.*, 1976).

Protein determination

In the preparation of component C4, the protein concentration of column eluates was followed by measuring the A_{280}. In the separation of the peptide chains by chromatography on hydroxyapatite, A_{280} could not be used, owing to interfering substances, and protein was measured by the method of Lowry *et al.* (1951) as modified by Hartree (1972).

Preparation of component C4

Frozen serum (500ml) was thawed and, after addition of 1ml of 2.5M-iPr$_2$*P*-F in propan-2-ol, was centrifuged at 23000g for 30min at 4°C. The supernatant was poured into 2 litres of water at 4°C containing 5mM-$CaCl_2$, 2.5mM-phenanthroline and 2.5mM-iodoacetamide. The pH was adjusted to pH7.4 with 1M-NaOH and 0.5ml of 2.5M-iPr$_2$*P*-F added. The suspension was stirred at 4°C for 2h and then centrifuged at 23000g for 30min, the euglobulin precipitate being taken for the preparation of component C1 (Gigli *et al.*, 1976). Then 1ml of 2.5M-iPr$_2$*P*-F was added to the supernatant and 0.5M-HCl added to adjust the solution to pH5.5. After stirring for 15min, the precipitate was removed by centrifugation and the supernatant adjusted back immediately to pH8.6 by adding 1M-Tris. NaCl was added to give 0.185. The solution from two preparations (total volume 5 litres) was passed through a column (7cm×27cm) of DEAE-Sephadex A-50 equilibrated with 0.01M-Tris/HCl (pH8.6)/0.185M-NaCl/0.02% NaN_3. The column was washed with 6.5 litres of the same buffer until the A_{280} of the eluate had fallen to 0.06. Then 600ml of 0.01M-Tris/HCl (pH8.6)/0.2M-NaCl/0.02% NaN_3 was run through the column, after which the remaining protein was eluted by a gradient formed by mixing 700ml of 0.01M-Tris/HCl (pH8.6)/0.2M-NaCl/0.02% NaN_3 and 700ml of 0.01M-Tris/HCl (pH8.6)/0.23M-NaCl/0.02% NaN_3. Elution with the latter buffer was continued until no further protein was eluted. The flow rate was about 300ml/h during the washing of the column and was decreased to about 100ml/h during the gradient elution. The fractions containing component C4 (Fig. 1*a*) were pooled, concentrated by ultrafiltration to 15ml and then iPr$_2$*P*-F was added to 1mM. This solution was passed through a column (5cm×90cm) of Sepharose 6B in 0.01M-Tris/HCl (pH8.6)/0.2M-NaCl/0.02% NaN_3. The fractions containing component C4 (Fig. 1*b*, Pool 1) were pooled and concentrated, iPr$_2$*P*-F was added to 5mM and the mixtures were stored at 2°C.

Separation of the polypeptide chains of component C4

The component-C4 solutions were dialysed against

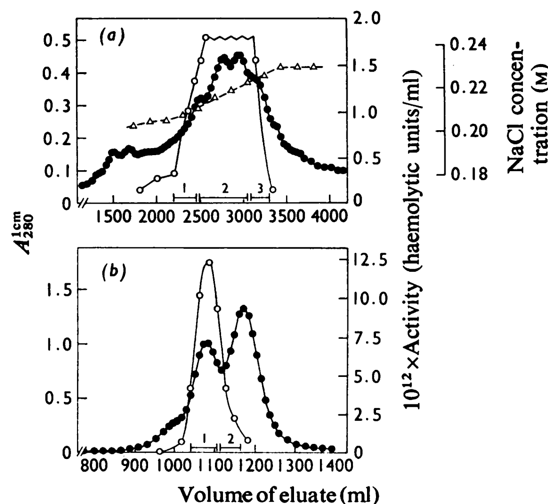


Fig. 1. *Chromatography of component C4*

(*a*) On a DEAE-Sephadex A-50 column (7cm×27cm), 5 litres of the pH5.5 supernatant was loaded and eluted with buffer containing 0.01M-Tris/HCl, pH8.6, and increasing concentration of NaCl. Active fractions were pooled as shown. ●, A_{280}; ○, component-C4 haemolytic units; △, NaCl concentration (M). (*b*) Pool 2 shown in Fig. 1(*a*) above was concentrated to 15ml, iPr$_2$*P*-F added to 1mM and the mixture loaded on the Sepharose 6B column (5cm×90cm) in buffer containing 0.01M-Tris/HCl (pH8.6)/0.2M-NaCl/0.02% NaN_3. ●, A_{280}; ○, component-C4 haemolytic units.

0.5 M-Tris/HCl, pH 8.6, then dithiothreitol was added to 20 mM and the mixtures were incubated at 37°C for 2h. Iodo[1-^{14}C]acetamide (67 μCi/mmol) was added to 50 mM. After standing for a further 30 min at room temperature (16–18°C), the solution was dialysed against 0.25 M-Tris/HCl, pH 8.6, at 4°C and then against 0.01 M-sodium phosphate, pH 6.4, containing 2% sodium dodecyl sulphate, and finally against the same phosphate buffer containing 0.1% sodium dodecyl sulphate. The peptide chains were separated on a hydroxyapatite column (1.4 cm × 35 cm) equilibrated with the same buffer. After loading and washing with this buffer the chains were eluted by a gradient formed from 500 ml of 0.2 M-sodium phosphate (pH 6.4)/0.1% sodium dodecyl sulphate and 500 ml of 0.5 M-sodium phosphate (pH 6.4)/0.1% sodium dodecyl sulphate as described by Moss & Rosenblum (1972). The eluate was screened by protein determination (Fig. 2) and by polyacrylamide-gel electrophoresis in buffers containing sodium dodecyl sulphate, and the fractions containing each of the separated chains were pooled and concentrated. Each was dialysed against 0.05 M-sodium phosphate, pH 7, with Dowex 1 (X8) resin in a dialysis bag also in the vessel, to facilitate the removal of the sodium dodecyl sulphate (Lenard, 1971).

An alternative technique was also used in which the γ-chain was separated from the α- and β-chains by fractionation on a column (2.6 cm × 95 cm) of Sephadex G-200 equilibrated with 6 M-guanidine hydrochloride, pH 7.0. Component C4 was reduced and alkylated, as described above, before dialysis into 6 M-guanidine hydrochloride. Separation of the α- and β-chains necessitated repeated recycling through a column (2.6 cm × 90 cm) of CL-Sepharose 6B.

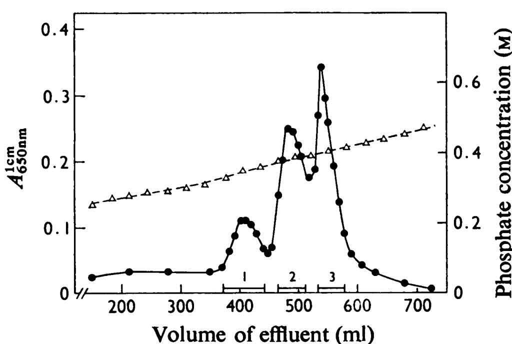


Fig. 2. *Chromatography of component-C4 peptide chains* Component-C4 polypeptide chains were separated by chromatography of 22 mg of reduced and alkylated component C4, on a column (35 cm × 1.4 cm) of hydroxyapatite in buffer containing 0.1% sodium dodecyl sulphate and increasing concentrations of sodium phosphate, pH 6.4. ●, Protein concentration by Lowry method (A_{650}); △, phosphate concentration.

Amino acid and carbohydrate analysis

Amino acid analysis was carried out as described by Reid (1974), with 1% phenol added to the constant-boiling HCl (Sanger & Thompson, 1963) and also 0.05% 2-mercaptoethanol if carboxymethylated proteins were hydrolysed. Hydrolysis in 3 M-toluene-*p*-sulphonic acid was used for determination of tryptophan (Liu & Chang, 1971) as well as hexosamines (Allen & Neuberger, 1975). For determinations of cystine and cysteine, component C4 was oxidized with performic acid (Hirs, 1956) and cysteic acid measured after hydrolysis. The peptide chains were reduced fully by incubation in 50 mM-dithiothreitol/6 M-guanidine hydrochloride/0.3 M-Tris/HCl, pH 8.6, for 3h at 37°C. Iodoacetamide was added to 250 mM and the solution left at 0°C for 1h. *S*-Carboxymethylcysteine was determined after hydrolysis. Neutral sugars and sialic acid were determined by g.l.c. after methanolysis (Clamp *et al.*, 1971). Samples containing 6–8 nmol of protein and 50 nmol of mannitol as internal standard were incubated at 90°C for 6h in 1.5 M-HCl in anhydrous methanol. The *O*-trimethylsilyl ethers of the methyl glycosides were detected in a Hewlett–Packard 5830 A gas chromatograph with a single column (200 cm × 0.32 cm) of 3% SE-30 Ultraphase on Chromosorb AW-DCMS 85–100 mesh.

N-Terminal amino acid determination and automatic sequencing

N-Terminal amino acids were determined by the dansyl procedure (Gray, 1972) and dansyl-amino acids resolved by t.l.c. on polyamide sheets (Woods & Wang, 1967). *N*-Terminal amino acid sequencing of the peptide chains after carboxyamidomethylation with iodo[1-^{14}C]acetamide was done in a Beckman 890C sequencer by using 0.1 M-Quadrol [0.1 M-*NNN′N′*-tetrakis-(2-hydroxypropyl)ethylenediaminetrifluoroacetate, pH 9.5], programmed as described by Brauer *et al.* (1975). Thiazolinones released were converted into phenylthiohydantoin derivatives, which were identified by t.l.c. and g.l.c. or amino acid analysis after hydrolysis with HI as described by Reid (1976). Release of radioactivity into the butyl chloride phase collected by the sequencer was determined by liquid-scintillation counting. A 0.3 ml portion was added to 15 ml of 1,4-dioxan containing 0.5% (w/v) 2,5-diphenyloxazole and 2% (w/v) naphthalene.

Results

Preparation of component C4

As in the preparation of component C1 (Gigli *et al.*, 1976), repeated addition of proteolysis inhibitors

was found necessary to obtain component C4 in good yield, and iPr$_2$P-F was the most effective inhibitor. The recoveries of component C4 at each step in the purification, as judged by haemolytic activity, were 80–90%, except from the final column of Sepharose 6B, when the recovery was about 50%; the lower values shown for the chromatography steps were not due to loss of activity but to selection of fractions to avoid impurities running close to the component C4 peak. Higher yields could be obtained if adjacent fractions were pooled and re-run, and this procedure was normally used (Table 1). The component C4 preparation obtained after fractionation on the DEAE-Sephadex A-50 and Sepharose 6B columns still contained 5–10% contaminating proteins as judged by polyacrylamide-gel electrophoresis. It was sufficiently pure to use for the isolation of the peptide chains on hydroxyapatite columns, as the contaminants were lost during the subsequent fractionation, but, for analysis of the whole protein as well as for purification of the chains by gel filtration, re-chromatography on a small DEAE-Sephadex A-50 column by using a similar gradient to that used before gave a product with barely detectable impurities (Plate 1). The isolated component C4 was stable at 4°C in solutions containing 0.05% NaN_3 over months, but freezing of solutions led to precipitation.

Reduction and alkylation of component C4

Component C4 was partially reduced under non-dissociating conditions, if desirable, followed by complete reduction in 6M-guanidine hydrochloride. If reduction was carried out in solutions containing 6M-guanidine hydrochloride or 2% sodium dodecyl sulphate, partial fragmentation of the α-chain was observed, giving rise to polypeptides with apparent mol.wts. of 42000 and 53000, as judged from electrophoresis in gels containing sodium dodecyl sulphate. The degradation might be due to a trace proteinase that is insensitive to heat and iPr$_2$P-F but sensitive to dithiothreitol; prior reduction under non-dissociating conditions prevents this fragmentation.

Isolation of the polypeptide chains of component C4

The γ-chain was separated easily from the α- and β-chains by exclusion chromatography in dissociating solvents, as it is of substantially lower molecular weight. Chromatography on columns of CL-Sepharose 6B in 6M-guanidine hydrochloride also gave a separation of the α- and β-chains if they were recycled repeatedly. This is, however, a tedious method, and only 50% of the protein gave α- and β-chains free of each other even after recycling seven times, as might be expected when their molecular weights differ by less than 20%.

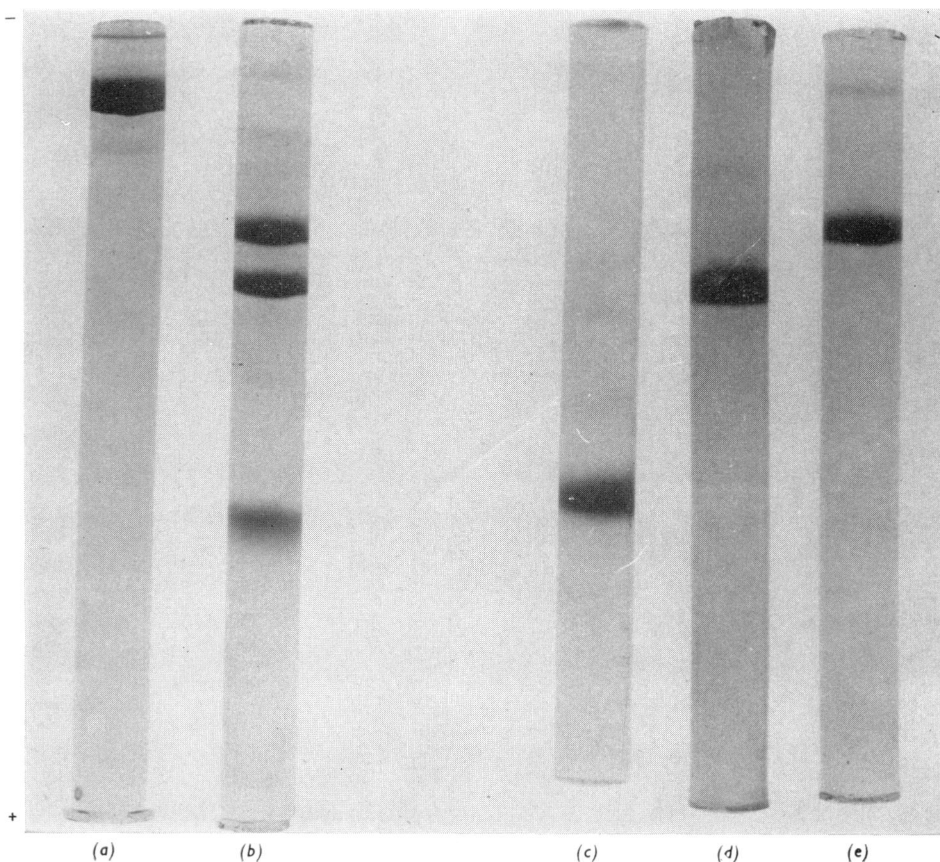
More effective was the method described by Moss & Rosenblum (1972) for separating protein subunits by using chromatography in sodium dodecyl sulphate solution on columns of hydroxyapatite. The peptide chains of component C4 dissociate fully in this solvent, and a satisfactory separation of the three chains was obtained in good yields (Fig. 2, Plate 1). The postulated molecular weight (Schreiber & Müller-Eberhard, 1974) should give protein yields of α-:β-:γ-chains in the proportions 1:0.85:0.35, and elution of the three chains from the hydroxyapatite column gave yields in the proportions 1:1:0.5. As the estimation of molecular weight was based only on the rate of movement during polyacrylamide-gel electrophoresis, and the α- and β-chains are not fully resolved on the hydroxyapatite column, these results are in reasonable agreement with the proposal that component C4 contains one of each of the three chains.

Table 1. *Fractionation of component C4 from serum*

The preparation was carried out with 995ml of serum. Yields in parentheses show total yields of component C4 from a preparation starting with 500ml of serum.

Procedure	Resulting fraction	Total volume (ml)	Total protein (mg)	Total activity (haemolytic units)	Specific activity (haemolytic units)	Total yield (%)
—	Serum	995	48110	4.39×10^{15}	9.12×10^{10}	100
Euglobulin precipitation	Supernatant	5143	47070	3.80×10^{15}	8.07×10^{10}	87 (91)
pH 5.5 precipitation	Supernatant	5198	43130	3.54×10^{15}	8.21×10^{10}	81 (80)
Chromatography on DEAE-Sephadex	Eluate pool 1*	259	62	2.00×10^{14}	3.23×10^{12}	
	pool 2	567	230	14.72×10^{14}	6.40×10^{12}	46 (64)
	pool 3	220	81	3.30×10^{14}	4.07×10^{12}	
Chromatography on Sepharose 6B	Eluate	68	62	7.18×10^{14}	1.16×10^{13}	16 (32)

* Eluate pools 1 and 3 from the DEAE-Sephadex column were re-run on the same column and active fractions combined with pool 2. Similarly, less pure fractions from the Sepharose 6B column were rerun and combined with the most active fraction. The reported yields represent the sum of high-specific-activity component C4 and the re-run fractions.



EXPLANATION OF PLATE 1

Electrophoresis of component C4 and its peptide chains

Electrophoresis on 5.6% polyacrylamide gel of component C4 and its peptide chains in buffers containing sodium dodecyl sulphate. Component C4, after chromatography twice on DEAE-Sephadex A-50 and once on Sepharose 6B, (*a*) unreduced and (*b*) reduced; (*c*) γ-chain, (*d*) β-chain, (*e*) α-chain from pools 1, 2 and 3 respectively shown in Fig. 2.

Amino acid and carbohydrate analysis

Analyses of the whole protein and the peptide chains of component C4 are given in Table 2. The overall pattern of amino acid residues is similar in the three chains, though there are some striking differences, such as the content of half-cystine and isoleucine in the γ-chain compared with that in the α- and β-chains. In general the amino acid contents of the α- and β-chains are rather similar to one another and obviously different from that of the γ-chain. The carbohydrate content of component C4 is 7.0% (Table 3), and analyses for the α- and β-chains show the α-chain to contain the larger part of the carbohydrate. No accurate estimate was obtained for the γ-chain, though some carbohydrate was present, and it appeared to be in similar content to that of the β-chains. Fucose was absent and glucose and galactosamine were probably only present in component C4 as contaminants.

N-Terminal amino acid sequences

N-Terminal amino acid determination by the dansyl method gave aspartic acid or asparagine for the α-chain, lysine for the β-chain and glutamic acid or glutamine for the γ-chain. By using the sequenator, the *N*-terminal amino acids were found to be α-chain asparagine, β-chain lysine and γ-chain glutamic acid. It was essential to remove the sodium dodecyl sulphate by extensive dialysis in the presence of Dowex 1 (X8) resin to get satisfactory results in the sequenator. The α-chain was soluble in 50mM-NH_3, the β-chain in 50% (v/v) acetic acid and γ-chain in 100mM-NH_3, but all three proteins showed a tendency to detach from the sequenator cup. This probably accounts for the incompleteness of the reaction, which became apparent after several steps of α- and

Table 2. *Amino acid composition of human component C4 and its three polypeptide chains*

Abbreviation: n.d., not determined. For details of analysis see the text.

Amino acid	Amino acid composition (Residues/100 residues)			
	C4	α-Chain	β-Chain	γ-Chain
Cys	1.47*	1.36‡	1.12‡	3.51‡
Asp	8.14	8.45	8.02	6.76
Thr	5.29	5.98	5.06	5.02
Ser	8.19	7.71	9.12	7.13
Glu	11.87	13.03	10.52	13.99
Pro	5.60	5.33	6.69	4.65
Gly	7.48	7.74	8.77	6.53
Ala	7.92	8.87	6.29	7.95
Val	7.71	7.32	8.45	7.88
Met	1.51†	1.57	1.38	1.40
Ile	3.09	3.17	4.22	1.89
Leu	11.05	11.85	10.55	10.46
Tyr	3.31	2.52	3.07	4.79
Phe	3.79	3.24	4.34	4.00
His	2.44	2.46	2.35	2.26
Lys	4.45	4.11	5.04	3.78
Arg	5.41	5.30	5.05	8.03
Trp	1.19	n.d.	n.d.	n.d.

* Measured as cysteic acid.
† Measured as methionine sulphone.
‡ Measured as *S*-carboxymethylcysteine.

Table 3. *Carbohydrate analysis of component C4 and of the α- and β-chains*

Sugar	Carbohydrate composition (residues/100 amino acid residues)		
	C4	α-Chain	β-Chain
Fucose	0	0	0
Mannose	1.4	1.2	1.6
Galactose	0.8	1.0	0.4
Glucose	≦0.07	<0.1	<0.1
Galactosamine	0	0	<0.1
Glucosamine	0.8	1.1	0.6
Sialic acid	1.0	1.5	0.7
Carbohydrate (% of total weight of protein)	7.0	8.6	5.6

Table 4. *Sequence analysis of the N-terminal residues of α-chain of component C4*

Component C4 α-chain (90nmol) was subjected to automated Edman degradation. Yields ranged from 6–10% in steps 1–5 down to 2–3% in steps 9–12, as estimated by amino acid analysis after hydrolysis in HI. Release of radioactivity was not detected. X is unidentifiable residue.

Residue no.	Method of identification of amino acid phenylthiohydantoin derivative			Proposed sequence
	G.l.c.	T.l.c.	Amino acid analysis	
1	—	Asn	Asx	Asn
2	Val	Val	Val	Val
3	—	Asn	Asx	Asn
4	—	Phe	Phe	Phe
5	—	Gln	Glx	Gln
6	—	Lys	Lys	Lys
7	—	—	—	X
8	Ile/Leu	Ile/Leu	Ile	Ile
9	—	Asn	Asx	Asn
10	—	Glu	Glx	Glu
11	—	Lys	Lys	Lys
12	—	Ile/Leu	Leu	Leu
13	—	—	—	X
14	—	—	—	X
15	—	Tyr	—	Tyr

Table 5. *Sequence analysis of the N-terminal residues of component-C4 β-chain*

Component-C4 β-chain (140nmol) was subjected to automated Edman degradation. Yields ranged from 11 to 4% as estimated by amino acid analysis after back-hydrolysis with HI. Release of radioactivity was not monitored. X is unidentifiable residue.

Residue no.	Method of identification of amino acid phenylthiohydantoin derivative: T.l.c.	Amino acid analysis	Proposed sequence
1	Lys	Lys	Lys
2	—	Pro	Pro
3	—	Arg	Arg
4	Leu/Ile	Leu	Leu
5	Leu/Ile	Leu	Leu
6	Leu/Ile	Leu	Leu
7	Phe	Phe	Phe
8	—	—	X
9	—	Pro	Pro

β-chain degradation. The presence of overlapping residues made the results for the α-chain after residue 10 somewhat uncertain, though the presence of a tyrosine residue at position 15 was clear (Table 4). On the β-chain eight *N*-terminal residues could be identified (Table 5). For automatic degradation of the γ-chain, the coupling step was repeated in step 1 and the cleavage step repeated in steps 1–3: 19 of the 20 *N*-terminal residues were identified (Table 6), including an *S*-carboxymethylcysteine residue at position 18 as shown by release of radioactivity because iodo[1-^{14}C]acetamide had reacted with SH groups after reduction of the component C4. The reduction was carried out with 20mM-dithiothreitol in the absence of denaturing agents, and it is likely that most of the cystine bonds split were interchain rather than intrachain bonds (Cecil & Wake, 1962). The yield of amino acid phenylthiohydantoins from the γ-chain fell abruptly after position 20, but the reason for this is not clear, as the next amino acid residue appeared to be arginine.

Table 6. *Sequence analysis of the first 20 N-terminal residues of component-C4 γ-chain*

Component-C4 γ-chain (95nmol) was subjected to automated Edman degradation. Yields ranged from 13% in the first step down to 3–4% in steps 15–18 and 8% in step 19, as estimated by amino acid analysis after back-hydrolysis in HI. X is unidentifiable residue.

Residue no.	Method of identification of amino acid phenylthiohydantoin derivative: T.l.c.	Amino acid analysis	Radioactivity released (c.p.m.)	Proposed sequence
1	Glu	Glx	43	Glu
2	Ala	Ala	31	Ala
3	—	Pro	35	Pro
4	Lys	Lys	40	Lys
5	Val	Val	45	Val
6	Val	Val	44	Val
7	Glu	Glx	42	Glu
8	Glu	Glx	46	Glu
9	Gln	Glx	51	Gln
10	Glu	Glx	47	Glu
11	—	—	54	X
12	—	Arg	55	Arg
13	Val	Val	54	Val
14	—	His	53	His
15	Tyr	Tyr	54	Tyr
16	—	Abu*	62	Thr
17	Val	Val	56	Val
18	—	Ala*	176	CmCys
19	Ile/Leu	Ile	93	Ile
20	Trp	—	70	Trp

* Thr and CmCys (*S*-carboxymethylcysteine) phenylthiohydantoin derivatives yield Abu (2-aminobutyric acid) and Ala, respectively, on hydrolysis in HI.

Discussion

To investigate the mechanism of activation of the early components of complement by antibody–antigen aggregates it is necessary to isolate in good yield and characterize the six proteins C1q, C1r, C1s, C2, C4 and C3 which are involved. The method described here takes the supernatant from the euglobulin precipitation at pH7.4 used for isolation of subcomponents C1q, C1r and C1s (Gigli *et al.*, 1976; Sim *et al.*, 1977). The precipitate formed when the pH of the supernatant is lowered to pH5.5 can be used for the isolation of component C3 (R. R. Porter & I. Gigli, unpublished work), and this second supernatant is the starting point for the isolation of component C4. As described in Gigli *et al.* (1976) and Sim *et al.* (1977), repeated addition of iPr$_2$*P*-F was necessary for good recoveries of component C4 and the other components in unactivated form. The purification of the components leads to the separation of the large amount of proteolysis inhibitors present in serum, and it appears likely that there is a continuous activation of small amounts of the proteolytic zymogens which remain as a persistent contaminant during purification.

The yield of component C4 obtained is satisfactory in view of the purification necessary and can be raised considerably by re-running of discarded

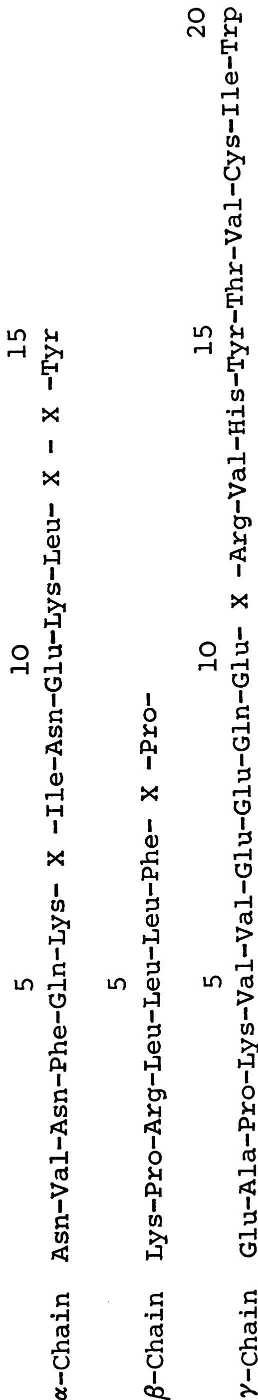


Fig. 3. *Summary of sequence data on the three polypeptide chains of component C4*

fractions. Difficulty was met in separation of the peptide chains in dissociating solvents such as 1 M-acetic acid (Bolotin *et al.*, 1976): 6 M-guanidine hydrochloride or sodium dodecyl sulphate solutions were necessary, and ion-exchange chromatography could not be used in these solutions. Fractionation by chromatography on hydroxyapatite columns in sodium dodecyl sulphate solution (Moss & Rosenblum, 1972) gave satisfactory resolution, but care had to be taken to remove all the detergent subsequently. Separation of the chains by exclusion chromatography in 6 M-guanidine hydrochloride was possible, and the guanidine hydrochloride was removed more easily and gave more soluble products, but the yields of the α- and β-chains free of each other were less. Both methods were used and the choice depends on the subsequent investigation to be carried out.

The present results confirm that component C4 contains one of each of three different peptide chains, in agreement with Schreiber & Müller-Eberhard (1974). A three-chain structure in proteins is exceptional, and there is a possibility that component C4 is synthesized as one or two chains which are hydrolysed subsequently. If so, the splitting is specific, as no heterogeneity was detected in the *N*-terminal sequences of any chain. Nagasawa & Stroud (1976) found the α- and γ-chains to have *N*-terminal residues of glutamic acid or glutamine, with that of the β-chain blocked, but in the present work investigation by dansylation and in the sequenator gave *N*-terminal residues for the α-, β- and γ-chains of asparagine, lysine and glutamic acid respectively. Carbohydrate is present on all three chains, with the highest amount on the α-chain.

It has been suggested that a structural gene for component C4 may map into the major histocompatibility locus (Teisberg *et al.*, 1976), because the gene coding for one of the peptide chains has arisen by duplication in this complex during evolution (Barnstable *et al.*, 1977). The mol.wt. of the γ-chain is similar to that of the Ia antigens, i.e. about 30000. The *N*-terminal 20 residues given here for the γ-chain, however, show no obvious similarity to the sequences reported for the 29000- and 33000-mol.wt. chains of human Ia antigen (Springer *et al.*, 1977). In particular, the sequence in the γ-chain positions 5–10, Val-Val-Glu-Glu-Gln-Glu, is unusual, and no similarities with other sequences have been found, including the *N*-terminal sequence of component C3 (Hugli *et al.*, 1975). There was no evidence for γ-carboxyglutamic acid residues in this sequence, glutamic acid being obtained in good yield in each position. As it is absent from the subcomponents of component C1, the Ca^{2+}-binding mechanism of complement proteins appears to be different from that of the blood-clotting enzymes (Davie & Fujikawa, 1975).

Extension of the structural studies will be necessary to establish the role of component C4 in the enzymically active $\overline{C4\text{–}C2}$ complex.

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References

Allen, A. K. & Neuberger, A. (1975) *FEBS Lett.* **60**, 76–80

Barnstable, C. J., Jones, E. A., Bodmer, W. F., Bodmer, J. G., Arce-Gomez, B., Snary, D. & Crumpton, M. J. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **41**, in the press

Bolotin, C., Morris, S., Tack, B. & Prahl, J. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1762

Brauer, A. W., Margolies, M. N. & Haber, F. (1975) *Biochemistry* **14**, 3029–3035

Budzko, D. B. & Müller-Eberhard, H. J. (1970) *Immunochemistry* **7**, 227–234

Cecil, R. & Wake, R. G. (1962) *Biochem. J.* **82**, 401–412

Clamp, J. R., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–334

Cooper, N. R. (1975) *J. Exp. Med.* **141**, 890–903

Curman, B., Östberg, L., Sandberg, L., Malmheden-Eriksson, I., Stålenheim, G., Rask, L. & Peterson, P. A. (1975) *Nature (London)* **258**, 243–245

Davie, E. W. & Fujikawa, K. (1975) *Annu. Rev. Biochem.* **44**, 799–829

Gigli, I., Porter, R. R. & Sim, R. B. (1976) *Biochem. J.* **157**, 541–548

Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138

Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427

Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621

Hugli, T. E., Vallota, E. H. & Müller-Eberhard, H. J. (1975) *J. Biol. Chem.* **250**, 1472–1478

Lachmann, P. J., Grennan, D. & Martin, A. (1975) *Nature (London)* **258**, 242–243

Lenard, J. (1971) *Biochem. Biophys. Res. Commun.* **45**, 662–668

Liu, T.-Y. & Chang, Y. H. (1971) *J. Biol. Chem.* **246**, 2842–2848

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Meo, T., Krasteff, T. & Shreffler, D. C. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4536–4540

Moss, B. & Rosenblum, E. N. (1972) *J. Biol. Chem.* **247**, 5194–5198

Nagasawa, S. & Stroud, R. M. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 654

Nelson, R. A., Jensen, J., Gigli, I. & Tamura, N. (1966) *Immunochemistry* **3**, 111–135

Patrick, A. R., Taubman, S. B. & Lepow, I. A. (1970) *Immunochemistry* **7**, 217–225

Reid, K. B. M. (1974) *Biochem. J.* **141**, 189–203

Reid, K. B. M. (1976) *Biochem. J.* **155**, 5–17

Reid, K. B. M. & Porter, R. R. (1975) *Contemp. Top. Mol. Immunol.* **4**, 1–22

Rosenfield, S. I., Ruddy, S. & Austen, K. F. (1969) *J. Clin. Invest.* **48**, 2283–2292

Sanger, F. & Thompson, E. O. P. (1963) *Biochim. Biophys. Acta* **71**, 468–471

Schreiber, R. D. & Müller-Eberhard, H. J. (1974) *J. Exp. Med.* **140**, 1324–1335

Sim, R. B., Porter, R. R., Reid, K. B. M. & Gigli, I. (1977) *Biochem. J.* **163**, 219–227

Springer, T., Kaufman, J., Siddoway, L., Gilhart, M., Mann, D., Terhorst, C. & Strominger, J. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **41**, in the press

Teisberg, P., Olaisen, B., Gedde-Dahl, T., Thorsby, E. & Åkesson, I. (1976) *Nature (London)* **264**, 253–254

Woods, K. R. & Wang, K.-T. (1967) *Biochim. Biophys. Acta* **133**, 369–370

World Health Organization (1968) *Bull. W.H.O.* **39**, 935–938