The Purification and Properties of the Second Component of Human Complement

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The second component of human complement (C2) was purified by a combination of euglobulin precipitation, ion-exchange chromatography, $(\text{NH}_4)_2\text{SO}_4$ precipitation and affinity chromatography. The final product was homogeneous by the criterion of polyacrylamide-gel electrophoresis and represents a purification of about 4000-fold from serum with 15–20% yield. Component C2 comprises a single carbohydrate-containing polypeptide chain, with an apparent mol.wt. of 102000; alanine is the *N*-terminal amino acid. The molecule is rapidly cleaved by activated subcomponent C1s with the loss of haemolytic activity to yield two fragments with apparent mol.wts. of 74000 and 34000. These fragments are not linked by disulphide bonds and can be easily separated. A second protein isolated during the purification of component C2 was identified by its haemolytic and antigenic properties as complement Factor B, the protein serving an analogous function to component C2 in the alternative pathway. The protein, which is also a single carbohydrate-containing polypeptide chain, has an apparent mol.wt. of 95000 and threonine as *N*-terminal amino acid. The amino acid analyses of component C2 and Factor B are compared.

The complement system causes lysis of antibody-coated cells and appears to be a major effector system of the immune response. The activation of the third component of complement (C3) is the central event of the complement system. It leads to lysis of target cells by the terminal complement components (C5–C9); the coating of target cells with component C3 fragments and their destruction by the cells of the mononuclear phagocytic system, and to the generation of small anaphylactic and chemotactic peptides. Activation is caused by the cleavage of the third component of complement *in vivo* and is effected by two complex proteolytic enzymes generated by two separate enzyme cascade systems, the classical and alternative pathways.

The classical pathway of complement activation is principally activated by antigen-antibody complexes. Subcomponent C1q binding to the complexes results in the sequential activation of two serine proteinases, subcomponents C1r and C1s. The activated subcomponent C1s in the C1 complex then cleaves the fourth component C4 and the second component C2, giving rise to the complex proteolytic enzyme C42, which serves as the component-C3 convertase (reviewed by Reid & Porter, 1975). The

Abbreviations used: the nomenclature of complement components and subcomponents is that recommended by the World Health Organization (1968); $\text{iPr}_2\text{P-F}$: di-isopropyl phosphorofluoridate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

alternative pathway is activated by certain non-immunoglobulin substances such as yeast cell-wall polysaccharide and bacterial endotoxins as well as by some immune aggregates. The exact method of activation of component C3 by the alternative pathway is not fully understood (for review see Götze & Müller-Eberhard, 1976), but the enzymically active protein in the component-C3 convertase, Factor B, has been isolated and partially characterized (Boenisch & Alper, 1970; Götze & Müller-Eberhard, 1971). It is an Mg^{2+} -dependent proteolytic enzyme and many of its properties are similar to those of component C2.

The identification of the structural genes for both component C2 and Factor B in the major histocompatibility complex on chromosome 6 in man has aroused additional interest in the relationship between the two molecules and to the possibility of their further function in the immune response (Fu *et al.*, 1974; Allen, 1974; Meo *et al.*, 1977). Any investigation of the structural relationship between the two molecules and study of assembly of the classical-pathway component-C3 convertase will require the purification of relatively large amounts of component C2; no such preparation has been available.

The purification of component C2 has been hampered by the low concentration of the protein in serum (about 20 mg/litre) and by the extreme susceptibility of the protein to proteolytic digestion during purification. The most extensive purification of com-

ponent C2, developed by Müller-Eberhard and co-workers (Polley & Müller-Eberhard, 1968), yielded small amounts of component C2 that, though apparently free of major contaminants, were highly unstable and required the addition of human serum albumin as stabilizing agent. The preparations were contaminated with the cleaved fragments of component C2 (Polley & Müller-Eberhard, 1968; Cooper, 1975). A preparation of stable guinea-pig component C2 was achieved by adsorption on sensitized erythrocytes coated with component C4. Although it produces a useful functional reagent, this approach is not applicable to large-scale preparations (Mayer *et al.*, 1970).

We now report the preparation of stable, apparently homogeneous, component C2 that is free of cleavage products. The preparation is achieved in good yield from serum by simple methods that could be scaled up to provide the larger amounts of material necessary for structural studies. Used in conjunction with the preparations of the C1 subcomponents and component C4 developed in our laboratory (Gigli *et al.*, 1976, 1977) this offers the possibility of studying the assembly of the classical-pathway component-C3 convertase.

Materials and Methods

Materials

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 and the serum stored at -20°C.

The sources of chemicals and other reagents have been described previously (Gigli *et al.*, 1976, 1977). All buffers contain 0.04% NaN_3 as preservative.

Haemolytic assay of components C2

Gelatin/veronal buffers were prepared as described by Nelson *et al.* (1966). Dilutions (0.5ml) of component C2 were incubated at 30°C with 0.5ml of erythrocytes ($10^8/\text{ml}$) sensitized by antibody and components C1 and C4 (Borsos *et al.*, 1970). The time of incubation was predetermined for each batch of cells such that maximum lysis was obtained. The time was usually 5 min. The haemolytic reaction was completed by the addition of 1.5ml of guinea-pig serum diluted 1:30 with 0.04M-EDTA in gelatin/veronal buffer; then 5.0ml of cold 0.15M-NaCl was added after incubation at 37°C for 1h and the mixture centrifuged at 1000g for 10min. The degree of lysis was measured from the A_{410} of the supernatant. Results were expressed in 50%-lysis units (H50 units) multiplied by the number of erythrocytes per ml (10^8).

Haemolytic assays of complement factor B

These were carried out in radial-diffusion plates by the method of Martin *et al.* (1976).

Preparation of affinity resins

Packed Sepharose 4B (25ml) was washed well with water; 3.5g of CNBr dissolved in 3.5ml of dimethylformamide was added to the Sepharose in a minimum amount of water and the mixture stirred until the reaction was completed. Throughout this time the temperature was kept at 20°C by the addition of crushed ice and the pH at 10–11 by the addition of 4M-NaOH (Cuatrecasas, 1970). When the reaction was complete the Sepharose was washed extensively with 0.1M-sodium phosphate (pH 7.8) and stirred overnight at 4°C in 100ml of the same buffer. This material is termed 'aged' CNBr-activated Sepharose 4B.

Sepharose 4B-bound component C4 was prepared by adding 30mg of component C4 in 35ml of 0.1M-sodium phosphate, pH 7.8, to the washed CNBr-treated Sepharose. The Sepharose 4B-bound component C4 was stirred overnight and then washed with 0.1M-sodium phosphate, pH 7.8. No component-C4 haemolytic activity could be detected in the washings.

Purification of component C2

Frozen serum (1000ml) was thawed and after addition of 1ml of 2.5M- $\text{iPr}_2\text{P-F}$ in propan-2-ol was centrifuged at 23000g for 30min at 4°C. The supernatant was poured into 4 litres of water at 4°C containing 5mM- CaCl_2 , 2.5mM-*o*-phenanthroline and 2.5mM-iodoacetamide (freshly recrystallized). The pH was adjusted to 7.4 with 1M-NaOH and 1ml of 2.5M- $\text{iPr}_2\text{P-F}$ added. The suspension was stirred at 4°C for 2h and then centrifuged at 23000g for 30min. The euglobulin precipitate was taken for the preparation of component C1 (Gigli *et al.*, 1976) and the supernatant was stored overnight at 4°C. On storage the pH of this pseudoglobulin fraction fell below 6.0 as a result of $\text{iPr}_2\text{P-F}$ hydrolysis, and the precipitate caused was removed by centrifugation at 23000g for 30min. Next 200ml of 0.4M-sodium phosphate, pH 6.0, 50ml of 0.2M-EDTA, pH 6.0, 2ml of 2.5M- $\text{iPr}_2\text{P-F}$ and 1ml of toluene were added to the supernatant (vol. 4.8 litres), and the pH was adjusted to 6.0 by addition of 4M-NaOH, if necessary. The sample was then loaded directly on to a column (10cm × 8cm) of CM-Sephadex C-50 equilibrated in 0.1M-sodium phosphate, pH 6.0. The column was washed with 1 litre of 0.1M-sodium phosphate, pH 6.0, containing 1ml of 2.5M- $\text{iPr}_2\text{P-F}$ and then developed with a linear gradient formed from 2 litres of 0.1M-sodium phosphate, pH 6.0, and 2 litres of 0.25M-sodium phosphate, pH 6.0, each containing

1 ml of 2.5 M- $i\text{Pr}_2\text{P-F}$. The column was finally washed with 1 litre of 0.4 M-sodium phosphate, pH 6.0, containing 1 ml of 2.5 M- $i\text{Pr}_2\text{P-F}$. The solutions were pumped through the column continuously at a rate of about 2 litres/h.

Active fractions from the CM-Sephadex column were pooled (2 litres), 1 ml of 2.5 M- $i\text{Pr}_2\text{P-F}$ was added and the mixture stored overnight. $(\text{NH}_4)_2\text{SO}_4$ was then added to 50% saturation (291 g/litre) and the suspension stirred for 1 h at 4°C, then centrifuged at 23000g for 30 min. The supernatant was decanted, filtered through Whatman no. 1 filter paper and $(\text{NH}_4)_2\text{SO}_4$ added (159 g/litre) to give 75% saturation. The suspension was stirred and left to warm to room temperature (15–20°C) before centrifugation at 23000g for 90 min at 20°C. The pellet was redissolved in about 50 ml of 0.4 M-sodium phosphate, pH 6.0, and stored overnight after the addition of 0.1 ml of 2.5 M- $i\text{Pr}_2\text{P-F}$. The cloudy suspension was centrifuged at 26000g for 30 min and the pellet discarded. The supernatant was passed through a column (30 cm $\times$ 4 cm) of Sephadex G-25 equilibrated in 5 mM-veronal buffer (pH 8.5)/0.5 mM- CaCl_2 /2.0 mM- MgCl_2 /0.04 M- NaCl and then applied to a column (5 cm $\times$ 2 cm) of 'aged' CNBr-activated Sepharose 4B equilibrated with the same buffer. The column was washed with this buffer until A_{280} of the eluate was zero, and then the component C2 eluted by a gradient formed from 250 ml of the veronal buffer and 250 ml of 0.4 M-sodium phosphate, pH 6.0. The active peak was pooled and equilibrated with 0.01 M-sodium phosphate, pH 7.8, by passage through a column (50 cm $\times$ 5 cm) of Sephadex G-25 in that buffer. The protein was then applied to a column (30 cm $\times$ 2.5 cm) of DEAE-Sephadex A-25 equilibrated in the same buffer. The column was developed by a linear gradient made from 500 ml of 0.01 M-sodium phosphate, pH 7.8, and 500 ml of 0.03 M-sodium phosphate, pH 7.8. The active fractions were pooled and their pH was lowered to 6.0 by addition of 3.0 M- H_3PO_4 . The pools were concentrated by adsorption on 1 ml of CM-Sephadex C-50 in a small column equilibrated in 0.1 M-sodium phosphate, pH 6.0, eluted with 0.4 M-sodium phosphate, pH 6.0, and stored at 4°C.

Purification of component C4

Component C4 was purified by the method of Gigli *et al.* (1977).

Purification of Factor B

Early fractions from the 'aged' CNBr-activated Sepharose columns, which contained most of this protein, were pooled and equilibrated with 0.01 M-sodium phosphate, pH 7.8. The sample was then loaded on a column (30 cm $\times$ 2.5 cm) of DEAE-Sephadex A-25 in the same buffer. The column was

washed extensively with the same buffer until the A_{280} of the eluate was zero and then the protein was eluted with a linear gradient formed from 300 ml of 0.01 M-sodium phosphate, pH 7.6, and 300 ml of 0.05 M-sodium phosphate, pH 7.6.

Electrophoresis in polyacrylamide gels

This was carried out in the Tris/glycine buffer system developed by Davis (1964). Gel electrophoresis in the presence of sodium dodecyl sulphate was performed by the method of Fairbanks *et al.* (1971) as described in Gigli *et al.* (1976) or with 7.5% (w/v) polyacrylamide slabs by the method of Laemmli (1970). Proteins were detected by staining with Coomassie Brilliant Blue and glycoproteins by the Schiff stain after periodate oxidation as described by Zacharius *et al.* (1969).

Amino acid analyses

These were done with a Locarte amino acid analyser. Proteins were hydrolysed with constant-boiling HCl, containing 5 mM-phenol, for 24, 48 and 72 h (Reid, 1974). Serine and threonine values were obtained from extrapolation back to zero time of hydrolysis, valine and isoleucine from the 72 h hydrolysis. Cysteine values were obtained from performic acid-oxidized samples (Hirs, 1956).

N-Terminal amino acid determinations

These were carried out by the dansyl procedure (Gray, 1972) in the presence of 1% sodium dodecyl sulphate; dansyl-amino acids were separated on polyamide thin-layer sheets (Woods & Wang, 1967) and identified by comparison with standards.

Results

Purification of component C2

After preliminary removal of component C1 from serum by euglobulin precipitation, 99% of the remaining serum proteins were removed by chromatography on CM-Sephadex C-50 (Fig. 1) and $(\text{NH}_4)_2\text{SO}_4$ precipitation. The resulting fraction was still a complex mixture of proteins, but by a series of purification steps utilizing gel filtration, ion-exchange chromatography and chromatography on hydroxyapatite it was possible to purify the component C2. A major contaminant, subsequently identified as complement Factor B, which is similar in molecular weight and charge to component C2 but present in great excess over component C2, did, however, prove extremely difficult to separate from component C2. The repeated chromatography necessary to remove this contaminant resulted in low yields of component C2.

Fig. 1. Chromatography of human serum pseudoglobulin on CM-Sephadex C-50

Human serum pseudoglobulin fraction (4.8-litres) containing 200ml of 0.4M-sodium phosphate, pH 6.0, 50ml of 0.1M-EDTA, 2ml of 2.5M- i -Pr₂P-F and 1ml of toluene was loaded on a column (10cm×8cm) of CM-Sephadex C-50 equilibrated in 0.1M-sodium phosphate, pH 6.0. The column was washed with 1 litre of 0.1M-sodium phosphate, pH 6.0, and eluted with a linear phosphate gradient as described in the Materials and Methods section. —, A_{280} ; ●, component-C2 haemolytic activity; ----, concentration of sodium phosphate (pH 6.0).

It was during attempts to develop an affinity column that would allow the rapid separation of component C2 from this contaminant that the curious property of component C2 adsorbing on 'aged' CNBr-activated Sepharose 4B was observed.

Since component C2 has been reported to form a reversible complex with component C4 (Müller-Eberhard *et al.*, 1967), we investigated the conditions under which this complex could be isolated. The complex was sufficiently stable to permit isolation by gel filtration of a mixture of components C2 and C4 on Sephadex G-200 in 5mM-veronal buffer (pH 8.5)/40mM-NaCl/0.6mM-CaCl₂/2.0mM-MgCl₂. The complex could then be dissociated in 0.1M-sodium phosphate, pH 6.0. Likewise, component C2 in partially purified preparation was adsorbed when passed through a column of Sepharose 4B-bound component C4 in the veronal buffer, whereas most of the protein was not adsorbed. The component C2 could then be eluted with the phosphate buffer (Fig. 2b). However, it was observed that exactly the same separation could be achieved under the same conditions by using a column of CNBr-activated Sepharose 4B 'aged' by stirring overnight under mildly alkaline conditions (Fig. 2a). Since no component-C4 haemolytic activity was detectable in the material applied to this column, component C4 cannot be necessary for the 'affinity' chromatography, although component C2 does not bind to untreated Sepharose 4B (Fig. 2c). The decay of the 'active' groups from CNBr-activated Sepharose 4B must therefore result in the formation of sites on the Sepharose 4B with affinity for component C2. This 'aged' CNBr-activated Sepharose 4B used as a routine in the preparation allowed the rapid separation of com-

ponent C2 from most of the Factor B. Factor B was retarded by the column, but not adsorbed completely under the conditions used (Fig. 3).

The final step of the purification of component C2, chromatography on DEAE-Sephadex A-25 (Fig. 4a), removed the remaining minor contaminants. Three pools were made from the active fractions eluted by the gradient. Pool 1 contained an apparently homogeneous protein giving a single band on electrophoresis in sodium dodecyl sulphate/polyacrylamide gels. A single band was also observed on electrophoresis at pH 8.3 in polyacrylamide gels in the absence of sodium dodecyl sulphate, and haemolytic assays performed on 1 mm slices of such gels showed the component-C2 activity to be coincident with this band. Pool 2 was similar, but contained a small trace of Factor B; pool 3, which contained about 15% of the recovered component-C2 activity, was heavily contaminated with Factor B.

The purification scheme is summarized in Table 1, and electrophoresis patterns in sodium dodecyl sulphate/polyacrylamide gels taken at various stages of the purification are shown in Plate 1. The pure component C2 represents a purification of about 4000-fold from serum. The final product is stable at neutral pH without the addition of proteolytic-enzyme inhibitors and only minor traces of the cleavage products reported in previous preparations (Polley & Müller-Eberhard, 1968). However, in the presence of activated subcomponent C1s the component C2 is cleaved rapidly into two fragments, C2a (apparent mol.wt. 74000) and C2b (apparent mol.wt. 34000), with complete loss of haemolytic activity. Both fragments can be identified on sodium dodecyl sulphate/polyacrylamide gels, though the smaller

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide gels (Laemmli, 1970) of samples (20 μ l) taken at various stages of the component-C2 purification
 1, Pseudoglobulin; 2, CM-Sephadex pool; 3, 75%-satd.-(NH₄)₂SO₄ pool; 4, 'aged' CNBr-activated Sepharose 4B pool; 5, concentrated DEAE-Sephadex pool 1.

EXPLANATION OF PLATE 2

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of component C2 and Factor B

(a) Run by the method of Fairbanks *et al.* (1971). 1, Purified component C2; 2, purified Factor B; 3, purified component C2+Factor B. (b) Run by the method of Laemmli (1970). 1, Component C2 incubated with purified subcomponent C1s, 37°C, 60min; (2) component C2 untreated.

Fig. 2. Chromatography of component C2

The protein precipitated at 75% satd. $(\text{NH}_4)_2\text{SO}_4$ was equilibrated with 5mM-veronal buffer, pH 8.5, 0.04M-NaCl, 2mM-MgCl₂, 0.5mM-CaCl₂. Samples (60ml) of the protein were loaded on to similar columns (5cm x 2cm) containing (a) 'aged' CNBr-activated Sepharose 4B, (b) component C4 coupled to Sepharose 4B, or (c) untreated Sepharose 4B, each equilibrated in the same buffer. The columns were washed and developed with increasing sodium phosphate concentrations (arrow indicates where gradient started) as described in the Materials and Methods section. —, A_{280} ; ●, component-C2 haemolytic activity. Letters a-t in (a) refer to fractions subjected to polyacrylamide-gel electrophoresis shown in Fig. 3.

fragment stains poorly (Plate 2). The fast weakly staining band seen in these gels is an artifact of unknown origin that is also seen on gels with no sample loaded.

Characterization of component C2

Component C2 is composed of a single polypeptide chain, as evident from the single band on sodium dodecyl sulphate/polyacrylamide gels run under reducing and non-reducing conditions. The apparent molecular weight estimated from the mobility of the protein in such gels compared with standards of known mol.wt. is 102000. Component C2 is a glycoprotein, staining with Schiff stain after periodate oxidation, although the carbohydrate content has

not yet been determined. The amino acid composition of component C2 is given in Table 2. A single *N*-terminal alanine residue was identified by the dansyl method.

Identification of a major protein co-purifying with component C2 as Factor B

A major contaminant that proved difficult to separate from component C2 by conventional procedures behaved like component C2 through most purification steps. As with component C2, this protein has a sole polypeptide chain of mol.wt. about 100000, but its apparent concentration in serum was about 200mg/litre. These properties suggested that it might be Factor B, the complement component serving an analogous function to component C2 in the alternative pathway. The protein was therefore purified from the early fractions from the 'aged' CNBr-activated Sepharose 4B column. A single chromatography step with DEAE-Sephadex in conditions almost identical with those used in the component C2 was sufficient (Fig. 4b). The fractions eluted by 0.02M-sodium phosphate, pH 7.6, were homogeneous with respect to electrophoresis in polyacrylamide gels (Plate 2). The protein is clearly resolved from component C2 in gels run as described by Fairbanks *et al.* (1971) (Plate 2), although the two proteins are resolved only poorly in 7.5% acrylamide gels run by the method of Laemmli (1970) (Fig. 3).

The contaminant protein was identified as complement Factor B by its haemolytic activity, the specific activity of the purified protein being comparable with that of purified Factor B prepared by Hunsicker *et al.* (1973). The protein also gave a single precipitation line in an Ouchterlony plate when tested with a specific anti-(Factor B) antiserum. Antisera raised against the purified protein gave a line of identity with the line produced with the anti-(Factor B) antiserum when tested against normal human serum. Finally, the amino acid composition of this protein is similar to that reported for glycine-rich β -glycoprotein (Table 2) (Boenisch & Alper, 1970), a serologically defined serum protein later identified with complement Factor B (Götze & Müller-Eberhard, 1971). The *N*-terminal amino acid of Factor B was threonine, not alanine as in component C2. Immunodiffusion analysis of purified component C2 and Factor B against antisera to each (Fig. 5) showed no antigenic cross-reactivity between the two proteins. The immunodiffusion analysis detected the small contamination of Factor B in this preparation of component C2.

Discussion

The purification scheme described allows the preparation of milligram quantities of stable com-

Fig. 3. Sodium dodecyl sulphate/polyacrylamide gels (Laemmli, 1970) of fractions taken from the 'aged' CNBr-activated Sepharose 4B column
Letters refer to fractions taken from the column shown in Fig. 2(a).

Fig. 4. Chromatography of (a) component C2 and (b) Factor B on DEAE-Sephadex A-25
(a) Protein eluted from the 'aged' CNBr-activated Sepharose 4B column (pool 2, Fig. 2a) was equilibrated with 0.1 M-sodium phosphate, pH 7.8, and loaded on to a column (30 cm $\times$ 2.5 cm) of DEAE-Sephadex A-25 equilibrated in the same buffer. The column was developed with increasing sodium phosphate concentration as described in the Materials and Methods section. —, A_{280} ; ●, component-C2 haemolytic activity; ----, concentration of sodium phosphate, pH 7.8. (b) Pool 1 from the 'aged' CNBr-activated Sepharose 4B column (Fig. 2a) was equilibrated with 0.01 M-sodium phosphate, pH 7.8, and loaded on to a column of DEAE-Sephadex A-25 (30 cm $\times$ 2.5 cm). The column was developed with increasing sodium phosphate concentration as described in the Materials and Methods section. Symbols are as in (a).

Table 1. *Purification of component C2 from serum*
For details of purification see the text.

Fraction	Volume (ml)	$10^{-13} \times$ Total activity (units)	Total protein (mg)	$10^{-11} \times$ Specific activity (units/mg)	Activity yield (%)
Serum	1000	19	56000	0.034	100
Pseudoglobulin	5500	18	55495	0.032	95
CM-Sephadex pool	2000	9.0	5780	0.16	47
75%-satd.-(NH ₄) ₂ SO ₄ pellet	80	6.1	576	1.1	32
'Aged' CNBr-activated Sepharose 4B pool	135	5.4	17.6	31	28
DEAE-Sephadex pools					
1	176	1.1	0.8	140	13
2	165	1.4	1.1	130	
3	165	0.5	0.6	80	

Table 2. *Amino acid compositions of human component C2 and Factor B*

Amino acid compositions of component C2 and Factor B were calculated from duplicate 24h, 48h and 72h HCl hydrolysates. Corrections were made for destruction of serine, threonine and tyrosine. Valine and isoleucine were estimated from the 72h hydrolysates. Cysteine was estimated from performic acid-oxidized samples (Hirs, 1956). Abbreviation: ND, not determined.

Amino acid	Amino acid composition (residues/100 residues)		
	Component C2	Factor B	Glycine-rich β -glycoprotein (Boenisch & Alper, 1970)
Cys	3.2	2.9	2.5
Asp	11.2	10.2	10.8
Thr	5.2	5.5	5.3
Ser	8.7	6.9	6.2
Glu	10.5	11.0	11.5
Pro	5.2	5.2	5.9
Gly	8.7	8.3	8.4
Ala	5.9	4.6	5.1
Val	6.5	7.4	7.7
Met	1.9	1.6	1.2
Ile	4.2	4.9	4.7
Leu	9.3	7.2	7.1
Tyr	2.7	5.6	4.3
Phe	4.1	2.7	2.7
His	2.7	2.6	2.5
Lys	4.7	7.2	7.4
Arg	5.3	5.1	5.4
Trp	ND	ND	1.3

ponent C2. The final yield of approx. 2mg from 1 litre of serum, with about 15% recovery of haemolytic activity, suggests a serum concentration for component C2 of only 15mg/litre. This is slightly lower than previous estimates (Polley & Müller-Eberhard, 1968; Glass *et al.*, 1976) and confirms component C2 to be one of the least abundant of the complement components in serum. In addition to the small amount of component C2 present in serum, a major difficulty in developing this purification scheme has been the avoidance of inactivation of

component C2 by proteolysis. This was also true in the purification of the subcomponents of C1 (Gigli *et al.*, 1976) and component C4 (Gigli *et al.*, 1977). Component C2 is even more unstable, being especially susceptible to proteolysis during column chromatography. The single most important measure to prevent proteolysis was the repeated addition of iPr₂P-F, especially during the early stages of the purification. Also, the preparation was carried out as quickly as possible, usually taking about 1 week, and the fractions were stored wherever possible at

Fig. 5. Immunodiffusion analysis of purified component C2 and Factor B

Well 1, rabbit anti-(human Factor B) antiserum; well 2, rabbit anti-(human component) C2 antiserum; well 3, purified human Factor B (0.1 mg/ml); well 4, purified human component C2 (DEAE-Sephadex pool 2, 0.1 mg/ml).

pH 6.0. All chromatography columns were recycled and repacked before use to ensure the removal of any protein bound to the columns and to allow rapid flow rates. DEAE- and CM-Sephadex columns were recycled as recommended by the manufacturers; the 'aged' CNBr-activated Sepharose 4B was washed thoroughly with 2M-NaCl between runs. With such precautions a final yield of 10–20% has been obtained as a routine.

The nature of the interaction between component C2 and 'aged' CNBr-activated Sepharose is unclear. It has, however, proved invaluable in the purification of component C2. The interaction appears to be specific, since greater than 30-fold purification is achieved in a single step, but other proteins do, however, bind to the resin (Fig. 3). The interaction is similar to an ion-exchange phenomenon in that it is decreased by higher salt concentrations and by a change in the pH, though the same separation is not achieved on CM- or DEAE-Sephadex under the same conditions. The exact mechanism of the CNBr activation of Sepharose and the loss of reactive groups on storage under mildly alkaline conditions is not fully understood, but it has been suggested that imidocarbonate groups are converted into carbamates (Joustra & Axen, 1975). The carbamates might function as weak absorption centres. Whether the fact that component C2 binds to and is eluted from the resin under conditions that allow formation and

dissociation of the component C2–C4 complex is purely coincidental, or whether the type of interaction in some way reflects the types of interaction involved in complex-formation, is not clear. The purification of component C2 by affinity chromatography on Sepharose 4B-coupled component C4 has been reported (Nagasawa & Stroud, 1977). Whether this purification is due to biospecific interaction or to the type of interaction described in the present paper is not known.

The properties of component C2 reported in the present work are similar to those reported in earlier studies. The mol.wt. of 102000 from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is slightly lower than the previous estimate based on measurements from sedimentation and diffusion properties (Polley & Müller-Eberhard, 1968). The stability of our preparation indicates that the instability of earlier preparations was due to contaminating proteolytic enzymes and not an intrinsic property of component C2. However, in agreement with the earlier studies we find that incubation of component C2 with activated component C1 results in the loss of component-C2 activity and the cleavage of the molecule. The two fragments that are formed are not linked by disulphide bonds and are easily separated. The molecular weights found for the larger fragment C2a (74000) and the smaller fragment C2b (34000), are similar to those reported by Nagasawa & Stroud (1977). They also found alanine as the *N*-terminal residue of the complete component-C2 molecule and also of fragments C2a and C2b.

The marked similarity between component C2 and Factor B of the alternative pathway has been discussed (Medicus *et al.*, 1976; Goodkofsky & Lepow, 1970). Their participation as the active enzymes in the respective component-C3 convertases and their similarity of molecular weight, heat-lability and Mg^{2+} -dependence have prompted the speculation that they are the products of gene duplication (Lachmann, 1975; Alper, 1976). The identification of allelic variants of both proteins, and the subsequent mapping of the structural genes of both proteins in the major histocompatibility complex (Meo *et al.*, 1977; Allen, 1974), has added considerable weight to this speculation. The similarity of the chemical and physical properties of the two proteins reported here can be taken as further evidence in favour of the suggestion, but the final proof must await the results of amino acid-sequence studies.

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