The separation of functionally distinct forms of the third component of human complement (C3)

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Complement component C3 prepared by the method of Tack & Prahl [(1976) *Biochemistry* 15, 4513–4521] was found to contain the following trace contaminants: C3b, haemolytically inactive C3 with intact α - and β -chains (C3u) and degraded C3 (apparent mol.wt. 140 000) with an intact β -chain but with a fragmented α -chain. The proportion of C3u in the C3 is increased on standing and by freezing and thawing. These contaminants could be separated from each other and from native C3 by chromatography on sulphated Sepharose. They have been characterized by their susceptibility to C3b inactivator in the presence of β_1 H, their ability to be cleaved by C3 convertase and their ability to form alternative-pathway C3 convertase in solution. Incubation of C3b or C3u with β_1 H and C3b inactivator resulted in cleavage of the C3 species; the α' -chain of C3b was cleaved to fragments of apparent mol.wts. 67 000 and 43 000, the α -chain of C3u was cleaved to fragments of apparent mol.wt. 75 000 and 43 000. Native C3 and degraded C3 were unaffected by incubation with β_1 H and C3b inactivator. C3u, unlike C3, was not cleaved to C3b by the classical- or alternative-pathway C3 convertase in solution. When C3b or C3 was incubated with factors B and $\bar{D}$, forming C3 convertase, the initial rate of factor-B cleavage was several orders of magnitude lower in the presence of C3 than in the presence of C3b. The slow rate observed for C3 could be decreased by preincubation with β_1 H and C3b inactivator or by rechromatography of the C3. The degraded C3 did not support factor-B cleavage by factor $\bar{D}$.

The third component of complement, C3, is the central protein of the system and is present in the serum in much higher amounts (about 1.2 mg/ml) than the other complement components. The activated form, C3b, is a subunit of C5 convertase in the classical pathway of activation and of both C3 and C5 convertase in the alternative pathway (Vogt *et al.*, 1978; Müller-Eberhard & Götze, 1972). When bound to aggregates or cells (Capel *et al.*, 1978; Fujita *et al.*, 1977; Law & Levine, 1977), C3b facilitates phagocytosis, as C3b receptors are present on macrophages and neutrophils (Huber *et al.*, 1968; Henson, 1969). C3a, the peptide re-

leased on activation, is an anaphylatoxin (Bokisch *et al.*, 1969; Hugli, 1975).

The most effective preparation of C3 in current use (Tack & Prahl, 1976) gives a substantially pure product in good yield, but when left in the cold or on freezing and thawing, haemolytic activity is lost without any obvious change in the peptide-chain structure, as shown by electrophoresis in polyacrylamide gels in sodium dodecyl sulphate-containing buffers. Small amounts of C3b and a degraded form of C3 are sometimes also present. We wished to investigate the structure of the alternative-pathway C3 convertase in solution, and particularly whether C3 or C3b could function equally effectively in this complex enzyme. The enzyme is formed by activation of the BC3b (or BC3) complex by the proteinase factor $\bar{D}$ (Fearon & Austen, 1975a; Müller-Eberhard & Götze, 1972), which is present in plasma in the activated form (Lesavre & Müller-Eberhard, 1978). In plasma, a

Abbreviations used: the nomenclature of complement components is that recommended by the World Health Organization (1968). VB, saline/veronal buffer; GVB, saline/veronal/gelatin buffer; DGVB, dextrose/saline/veronal/gelatin buffer (full compositions are given in the text).

fourth protein, properdin P, also associates with BC3 or BC3b and factor D; its role appears to be to stabilize the complex (Schreiber *et al.*, 1975). In the activation, factor B is split into two fragments, Ba and Bb, of 30000 and 60000 mol.wt. respectively (Kerr, 1979), with the proteolytic site in the larger fragment (Medicus *et al.*, 1976; Christie *et al.*, 1980). The C3 convertase formed catalyses conversion of C3 into C3b; the product can then function as a subunit in the formation of additional C3 convertase.

There is a major difficulty in deciding whether both C3 and C3b can form C3 convertase in the presence of factor B and factor D, as trace contamination of C3 with C3b will lead to formation of more C3b, and hence C3 convertase activity, even if C3 alone cannot function as a subunit of the convertase (Fearon & Austen, 1975*b*; Schreiber *et al.*, 1978).

It was necessary to develop a method of fractionation of apparently pure C3 to remove traces of other products of C3. This has been done by chromatography on sulphated Sepharose, which separated C3, C3b, one or possibly two forms of haemolytically inactive C3, and a partially degraded form of C3. These proteins have been characterized by their chain structure, haemolytic activity, ability to form C3 convertase and their susceptibility to the complex control proteinase formed from C3b inactivator and $\beta_1\text{H}$. This proteinase, in which C3b inactivator is the catalytic component and $\beta_1\text{H}$ is an essential cofactor, hydrolyses C3b but not C3, and is believed to be responsible for the destruction of C3b *in vivo* (Pangburn *et al.*, 1977; Harrison & Lachmann, 1980). The results obtained show that initial rates of factor B splitting and parallel development of C3 convertase activity in the presence of C3 or C3b and factor D is several orders of magnitude lower for C3 than for C3b.

Materials and methods

Materials

Outdated human plasma was obtained from the John Radcliffe Hospital, Oxford, U.K. Chemicals were obtained as follows: CM-Sephadex C50, Sephadex G-75 and Sepharose CL 4B from Pharmacia, Hounslow, Middx., U.K.; $(\text{NH}_4)_2\text{SO}_4$ and dimethylformamide from Fisons, Loughborough, Leics., U.K.; acetone and dichloromethane from May and Baker, Dagenham, Essex, U.K.; chlorosulphonic acid from BDH Ltd., Poole, Dorset, U.K.; and pyridine from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.

Purification of complement components

C3 was purified from outdated plasma by the method of Tack & Prahl (1976) or by modification

of this method as described by Sim *et al.* (1981). $\beta_1\text{H}$ was obtained as a by-product of the C3 preparation and purified to homogeneity as described by Sim & Sim (1981). C3b inactivator was prepared by the method of Crossley & Porter (1980). C4 and C1s were purified by the methods of Gigli *et al.* (1976, 1977).

C2 and factor B were prepared from serum by the method of Kerr & Porter (1978) as modified by Kerr (1979). Guinea-pig C2 was prepared by using the method for human C2 with minor modifications. Factor D was purified by using the same initial steps: euglobulin precipitation and chromatography on CM-Sephadex C50 as described for the C2 and factor-B preparations, the factor D being eluted from the CM-Sephadex column by a second linear salt gradient formed from 2 litres of 0.25 M-sodium phosphate, pH 6.0, and 2 litres of 0.4 M-sodium phosphate, pH 6.0, each containing 0.5% (w/v) benzamidine hydrochloride and 0.04% (w/v) NaN_3 . Active fractions from the CM-Sephadex column were pooled (2 litres), $(\text{NH}_4)_2\text{SO}_4$ added to 50% saturation (291 g/litre) and the suspension stirred for 2 h at 4°C, then centrifuged at 18000 g for 30 min at 4°C. The supernatant was filtered with Whatman no. 1 filter paper and $(\text{NH}_4)_2\text{SO}_4$ added to 70% saturation (125 g/litre). The suspension was stirred for 2 h at room temperature (15–20°C) and then centrifuged at 18000 g for 2 h at 20°C. The pellet was redissolved in 6 ml of 0.1 M-sodium phosphate, pH 6.0, loaded on to a column (150 cm $\times$ 2.5 cm) of Sephadex G-75 equilibrated in 0.05 M-sodium phosphate, pH 6.0, and the protein eluted with 0.05 M-sodium phosphate, pH 6.0. The fractions containing factor D haemolytic activity were pooled and concentrated by ultrafiltration with a UM2 membrane in an Amicon Diaflo apparatus. The concentrate (10 ml) was frozen at –70°C. The factor D prepared by this method was usually homogeneous by the criterion of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and the yield of haemolytic activity was routinely 20%. In a few preparations, a contaminant with mobility identical with that of factor D in sodium dodecyl sulphate/polyacrylamide gels run under non-reducing conditions, but of slower mobility on the same gels under reducing conditions, was observed. This contaminant could be removed as described by Johnson *et al.* (1980).

C3b was prepared by incubation of C3 (8 mg) with factor B and factor D (C3/B/D, 50:5:1, by weight) in 5 mM-veronal buffer (pH 7.5)/0.15 M-NaCl/1.0 mM-MgCl₂/0.3 mM-CaCl₂ at 37°C for 1 h. The incubation mixture was then dialysed against 0.1 M-sodium phosphate (pH 6.0)/0.04% (w/v) NaN_3 and loaded on to a column (8 cm $\times$ 2 cm) of CM-Sephadex C50 equilibrated in the same buffer. The C3b was not retarded by the column and was

separated from C3, factor B, Bb, Ba and factor D. The latter were eluted by adding 0.5 M-NaCl to the buffer (Crossley & Porter, 1980).

Buffers for cell preparation and haemolytic assays

Gelatin/veronal buffers (GVB) were prepared as described by Nelson *et al.* (1966): GVB is composed of iso-osmotic 5 mM-veronal buffer (pH 7.5)/0.1% (w/v) gelatin; DGVB comprises equal volumes of GVB and 5% glucose containing 0.5 mM-MgCl₂ and 0.15 mM-CaCl₂; GVB-EDTA (9:1) comprises GVB and 0.1 M-EDTA, pH 7.5, in the ratio 9:1 (v/v); GVB-EDTA (6:4) contains GVB and 0.1 M-EDTA, pH 7.5, in the ratio 6:4 (v/v); VB is composed of iso-osmotic 5 mM-veronal buffer, pH 7.5, containing 1.0 mM-MgCl₂ and 0.3 mM-CaCl₂.

Preparation of EAC43 cells

Sheep erythrocytes (1×10^8 cells/ml) sensitized with antibody and complement components C1 and C4 (EAC14 cells; Borsos & Rapp, 1967) were incubated with purified human C2 (400 ng/ml) and C3 (5 µg/ml) in DGVB for 5 min at 30°C and then chilled on ice. The cells were centrifuged at 1000 g for 5 min at 4°C and resuspended in GVB-EDTA (9:1). The cells were then washed once with the same buffer, incubated at 37°C for 15 min and washed again with GVB-EDTA (9:1). After a second incubation at 37°C for 15 min, the cells were washed three times in DGVB and resuspended in DGVB containing 40 units each of streptomycin and penicillin/ml before storage at 4°C.

Haemolytic assays for C3, factor B and factor D

C3 was assayed by using EAC14 cells by the method of Tack & Prahl (1976) as modified by Kerr (1980).

Factor B was assayed by using EAC43 cells as follows. Factor B in 0.1 ml of DGVB was incubated with 0.1 ml of EAC43 cells (1×10^8 cells/ml in DGVB) and 0.1 ml of factor D (6 µg/ml in DGVB) for 30 min at 30°C. Guinea-pig serum [0.3 ml; diluted 1:30 in GVB-EDTA (6:4)] was added and the incubation continued for 1 h at 37°C. After addition of 1.0 ml of 0.15 M-NaCl the cells were pelleted by centrifugation at 1000 g and 4°C for 7 min and the degree of lysis determined from the A_{412} of the supernatant.

Factor D was assayed by a similar procedure, the initial incubation containing factor D in 0.1 ml of DGVB, EAC43 cells (0.1 ml) and 0.1 ml of factor B (30 µg/ml in DGVB).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis in 7.5 and 10% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulphate was performed as described by Laemmli

(1970). When reduction of the samples was necessary, they were incubated with 40 mM-dithiothreitol/4 M-urea/1% (w/v) sodium dodecyl sulphate/0.05 M-Tris/HCl, pH 8.0, at 37°C for 10 min. After staining with Coomassie Brilliant Blue, gels were scanned with a Joyce-Loebl densitometer.

Preparation of sulphated Sepharose

Packed Sepharose CL 4B (100 ml) was washed with 3×200 ml of acetone on a Buchner funnel with Whatman no. 1 filter paper, followed by 2×200 ml of dimethylformamide (dried over anhydrous Na₂SO₄). The Sepharose was then transferred to a flask containing 200 ml of dry dimethylformamide and left to equilibrate for 24 h. The dry Sepharose was filtered, resuspended in dry dimethylformamide and transferred to a silicone-treated beaker. The suspension was stirred gently and kept at -5°C by using an ethanol/solid-CO₂ bath during the preparation. Dry pyridine (4 ml) was added dropwise and then a chilled mixture of 3 ml of chlorosulphonic acid in 6 ml of dichloromethane was added dropwise over 1½ h. The mixture was stirred for a further 30 min and then left for 30 min. The supernatant was decanted off and 200 ml of ice-cold ethanol added to the gel. The sulphated Sepharose, after being filtered on a Buchner funnel, was transferred to 200 ml of cold 50% (v/v) ethanol/50% (v/v) 0.2 M-NaHCO₃/0.02 M-Na₂CO₃, pH 9.0, to neutralize any remaining acid. After extensive washing in 0.2 M-NaHCO₃/0.02 M-Na₂CO₃, pH 9.0, the sulphated Sepharose was equilibrated in 25 mM-Tris/phosphate (pH 6.1)/60 mM-NaCl/0.04% (w/v) NaN₃. Sulphur analysis showed that the sulphated Sepharose contained 8% (w/w) sulphur.

Results

Identification of structurally distinct forms of C3 as trace contaminants in C3 preparations

Sodium dodecyl sulphate/polyacrylamide gels of two preparations of C3 purified by the method of Tack & Prahl (1976) are shown in Fig. 1. Under non-reducing conditions, in addition to C3, a degradation product of C3 (apparent mol.wt. 140 000) was observed as a trace contaminant. Under reducing conditions, C3 was seen as two polypeptide chains, C3α and C3β. Polypeptides with apparent mol.wts. of 43 000, 27 000, 25 000 and 23 000, presumably derived from the degraded species, and C3α' from C3b, were also observed.

When C3 was incubated with the components of the classical-pathway C3 convertase, cleavage to C3a and C3b occurred with the formation of the C3α' chain. When all the haemolytic activity of the C3 had been lost, some C3α chain remained. This uncleavable material, which is haemolytically in-

Fig. 1. Resolution of complement component C3

Sodium dodecyl sulphate/7.5%-polyacrylamide gels of two preparations of C3. NR, non-reduced samples; R, reduced samples. The second track of each pair shows the C3 after incubation with classical-pathway C3 convertase for 1 h at 37°C (C3/C4/guinea-pig C2/C1s, 1000:20:10:1, by weight).

Fig. 2. Separation of C3 species on sulphated Sepharose

Separation of a mixture of C3 containing a high proportion of degraded C3, C3b and C3u obtained by freezing C3 (20 mg of protein) on a column (25 cm × 2 cm) of sulphated Sepharose in 25 mM-Tris/phosphate (pH 6.1)/60 mM-NaCl/0.04% NaN₃, with an [NaCl] gradient from 0.06 M to 0.8 M. —, A_{280} ; ----, [NaCl]; O, C3 haemolytic activity expressed as $Z = -\ln(1 - Y)$, where Y is the fraction of cells lysed.

active, we have termed 'C3u' (u denoting uncleavable by C3 convertase).

The C3 preparation thus appears to contain four forms of the C3 molecule: native C3 and traces of C3b, C3u and degraded C3.

Separation of the forms of C3

A mixture of the four forms of C3 was prepared by mixing a preparation of C3 containing an unusually high proportion of degraded C3 with C3 inactivated by freezing and thawing (C3u) and purified C3b. The mixture, containing approx. 20 mg of protein, was dialysed against 25 mM-Tris/

phosphate buffer (pH 6.1)/60 mM-NaCl/0.04% (w/v) NaN₃ and, after centrifugation to remove the small amount of precipitate, the sample was applied to a column (25 cm × 2 cm) of sulphated Sepharose equilibrated with this buffer. The column was washed with 75 ml of the same buffer and then developed with a linear gradient formed from 150 ml of this buffer and 150 ml of 25 mM-Tris/phosphate (pH 6.1)/0.8 M-NaCl/0.04% (w/v) NaN₃. The elution profile is shown in Fig. 2. The result of polyacrylamide-gel electrophoresis of fractions taken across the column elution profile is shown in Fig. 3. The degraded form of C3 was not bound to the column.

Fig. 3. C3 fractions after separation on sulphated Sepharose

Sodium dodecyl sulphate/polyacrylamide gel of fractions taken across the sulphated-Sepharose-column elution profile shown in Fig. 2. The samples were reduced before electrophoresis. L, a sample of the material loaded on to the column.

C3b eluted at the beginning of the salt gradient, followed by haemolytically active C3 and finally C3u.

Reaction with fluid-phase alternative-pathway C3 convertase

The reaction of C3 and C3u with alternative-pathway C3 convertase was investigated by incubation of samples (25 μ g) with factor B (5 μ g), factor $\bar{D}$ (1.5 μ g) and C3b (1 μ g) in 1.5 ml of VB at 37°C. Samples were removed and, after reduction, subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The cleavage of C3 to C3b and of factor B to Bb was measured by densitometric scanning of the gels. As shown in Fig. 4, C3 was cleaved in the presence of factor B, factor $\bar{D}$ and C3b, but not in the absence of either factor B or factor $\bar{D}$. C3u was not cleaved, therefore the lack of cleavage by the alternative-pathway C3 convertase correlated with lack of cleavage by the classical-pathway C3 convertase. In the presence of C3, more cleavage of factor B was seen than in the presence of C3u, presumably because of an increase in the amount of C3b in the incubation as C3 was cleaved.

Cleavage by C3b inactivator and β_1H

Equal volumes of fractions from the sulphated Sepharose column were incubated with purified β_1H and C3b inactivator ($\beta_1H/C3bINA$, 5:1, w/w; the proportion by weight of C3 was equal to or less than that of the β_1H) for 1 h at 37°C and the products analysed, after reduction, on a polyacrylamide gel (Fig. 5). C3 and the degraded C3 were unaffected by the incubation. The α' -chain of C3b was cleaved to two fragments of apparent mol.wts. 67000 and 43000. C3u was also cleaved by β_1H and C3b

Fig. 4. Reaction of C3 and C3u with alternative-pathway C3 convertase

C3 or C3u (25 μ g) was incubated with 5 μ g of factor B, 1.5 μ g of factor $\bar{D}$ and 1 μ g of C3b in 1.5 ml of VB at 37°C. Samples were removed at various times and, after reduction, run on a sodium dodecyl sulphate/10%-polyacrylamide gel. The percentage of uncleaved B (a) and the percentage of C3 α chain remaining (b) were determined by densitometric scanning of the gels. Δ , C3; $\circ$, C3u; $\bullet$, C3 or C3u in the absence of factor $\bar{D}$ or factor B.

Fig. 5. Reaction of C3 species with β_1H and C3b inactivator

Sodium dodecyl sulphate/7.5%-polyacrylamide gel of fractions taken across the sulphated-Sephadex-column elution profile after incubation for 1 h at 37°C with C3b inactivator and β_1H . The samples were reduced before electrophoresis. The fractions shown are identical with those in Fig. 3. Equal volumes (30 μ l) of the fractions were used, giving a ratio β_1H /C3bINA, 5:1, w/w; the proportion by weight of C3 was equal to, or less than, that of the β_1H . A, β_1H ; B, C3b inactivator.

inactivator, the α -chain being cleaved to fragments of apparent mol wts. 75 000 and 43 000. The α -chain fragment of apparent mol.wt. 43 000 migrated in a manner identical with that shown by one of the polypeptide chains of the degraded C3.

Roles in formation of the fluid-phase alternative-pathway C3 convertase

The ability of C3, degraded C3 and C3b to support factor B cleavage by factor $\bar{D}$ is shown in Fig. 6. Samples from the incubation mixtures, 25 μ g of C3, degraded C3 or C3b, 1.5 μ g of factor B and 1.5 μ g of factor $\bar{D}$ in 1.5 ml of VB at 37°C, were run, after reduction, on polyacrylamide gels, and the factor B cleavage was measured by densitometric scanning of the gels. C3b supported the rapid cleavage of factor B to Ba and Bb. On extended incubation, native C3 also supported the cleavage of factor B; the rate was slower than for C3b. No cleavage was seen for either C3 or C3b in the absence of factor $\bar{D}$. The degraded C3 did not support the cleavage of factor B by factor $\bar{D}$.

When C3 is chromatographed on sulphated Sepharose, the C3b peak tends to trail into that of the native C3. Rechromatography of native C3 on the column resulted in an increase in the specific haemolytic activity on the C3, but the ability to support factor B cleavage in the presence of factor $\bar{D}$ was decreased. However, it has not been possible to purify the C3 to remove the ability to support factor B cleavage completely.

When native C3 (20 μ g) was incubated for 2 h at 37°C in 25 mM-Tris/phosphate (pH 6.1)/0.2 M-

Fig. 6. Reaction of C3 species with factors B and $\bar{D}$. C3, degraded C3 or C3b (25 μ g) was incubated with 1.5 μ g of factor B and 1.5 μ g of factor $\bar{D}$ in 1.5 ml of VB at 37°C. Samples were removed at various times and, after reduction, run on a sodium dodecyl sulphate/10%-polyacrylamide gel. The percentage of factor B cleaved was determined by densitometer scanning of the gel. ●, C3b; ○, C3; △, degraded C3 and also C3b or C3 in the absence of factor $\bar{D}$.

NaCl with C3b inactivator (2 μ g) and β_1H (2 μ g) before incubation at 37°C in 25 mM-Tris/phosphate (pH 7.5)/0.15 M-NaCl/2 mM-CaCl₂/7 mM-MgCl₂ with factor B (15 μ g) and factor $\bar{D}$ (0.3 μ g), no cleavage

Fig. 7. Effect of preincubation of C3 with β_1H and C3b inactivator on factor B cleavage

C3 (20 μ g) was incubated for 2 h at 37°C in 25 mM-Tris/phosphate (pH 6.1)/0.2 M-NaCl with C3b inactivator (2 μ g) and β_1H (2 μ g) before incubation at 37°C in 25 mM-Tris/phosphate (pH 7.5)/0.15 M-NaCl/2 mM-CaCl₂/7 mM-MgCl₂ with factor B (15 μ g) and factor D (0.3 μ g). In the second incubation the ratio C3/B/D/ β_1H /C3b inactivator was 50:50:1:5:5 by weight. Samples were removed from the second incubation at various times and, after reduction, run on a sodium dodecyl sulphate/10%-polyacrylamide gel. The percentage of factor B cleaved was determined by densitometric scanning of the gel. O, C3 alone in the first incubation; ●, C3 with β_1H ; Δ , C3 with β_1H and C3b inactivator.

of factor B occurred (Fig. 7). Since C3b inactivator and β_1H have no apparent effect on native C3, but do cleave C3b, it is possible that the cleavage of factor B by factor D in the presence of native C3 is due to contamination with C3b.

Discussion

During investigation of the role of C3 in the alternative-pathway C3 convertase, it was found that C3 prepared by the method of Tack & Prahl (1976) contained traces of C3b, C3u and degraded C3. These species and native C3 can be isolated by chromatography of the C3 preparation on sulphated Sepharose (Figs. 2 and 3).

The degraded C3 appears, after reduction, on sodium dodecyl sulphate/polyacrylamide gels to

have an intact β -chain and a much-fragmented α -chain. The presence of the band at 43 000 mol.wt. suggests that degraded C3 is formed by the action of C3b inactivator and β_1H on C3; however, additional proteolytic cleavage of the C3 α -chain must also occur to produce the smaller fragments observed. The degraded C3 has an apparent mol.wt. on sodium dodecyl sulphate/polyacrylamide gels of 140 000, but runs just before C3 on Sephadex G-200; it is therefore uncertain whether the molecule has a complete α -chain or whether a fragment is missing. The 43 000-mol.wt. peptide is strongly labelled with ¹²⁵I when the chloramine-T method is used (E. Sim, unpublished work), thus to obtain an accurate specific radioactivity for C3, the degraded C3 must be removed. The degraded C3 is not affected by incubation with β_1H and C3b inactivator or with C3b, factor B and factor D, nor will it support cleavage of factor B by factor D.

C3u is haemolytically inactive and is uncleavable by the classical- and alternative-pathway C3 convertases in solution. It is identical with C3 on electrophoresis on sodium dodecyl sulphate/polyacrylamide gels, and for this reason its presence in C3 preparations is not readily detected. The sodium dodecyl sulphate/polyacrylamide gels indicate that the polypeptide-chain structure of C3u is the same as that of C3, thus the difference in properties of the two species must result from a difference in conformation. The proportion of C3u in the C3 is increased on standing, by ultrafiltration and by freezing and thawing. The C3 is particularly susceptible to inactivation when frozen in veronal buffers. C3 treated with amines loses its haemolytic activity, acquires a free thiol group and becomes less well cleavable by C3 convertase (von Zabern *et al.*, 1980, 1981; Tack *et al.*, 1980). Thus amine-treated C3 appears similar to C3u; this is in agreement with the correlation in properties reported by von Zabern *et al.* (1980, 1981) for amine-treated C3 and C3 that had been frozen slowly. Neither C3u nor hydroxylamine-treated C3 undergoes the denaturation cleavage described by Sim & Sim (1981). Both C3u and hydroxylamine-treated C3, like C3b, are cleaved by β_1H and C3b inactivator (Sim & Sim, 1981; Crossley & Porter, 1980).

The production by C3 convertase of C3b in solution is the first stage in the activation of the alternative pathway. The C3 convertase is formed when factor B, bound to C3b or possibly C3, is cleaved by factor D. It is difficult to determine whether C3b is the only C3 species capable of forming C3 convertase or whether C3 shares this activity, since either trace contamination of C3 with C3b or a low intrinsic activity would result in the formation of C3b and hence more convertase. For C3 and C3b purified with sulphated Sepharose, the initial rate of factor B cleavage by factor D is many

orders of magnitude lower for C3 than for C3b. Since the C3b runs just before C3 on the column and tends to trail, it is possible that the purified C3 still contains traces of C3b. This is supported by the observations that rechromatography of the C3 results in a decrease in the initial rate of factor B cleavage, and that, in confirmation of Schreiber *et al.* (1978), preincubation of the C3 with $\beta_1\text{H}$ and C3b inactivator abolishes factor B cleavage. Thus it appears that any intrinsic ability of C3 to function as a component of the convertase must be very much less than that of C3b.

The role of C3u in the formation of C3 convertase is unclear. The haemolytically inactive C3 that is eluted from the sulphated Sepharose at the end of the salt gradient appears to be heterogeneous. The material eluted from the column usually contains more haemolytically inactive C3 than the original sample, suggesting that, in addition to the inactive C3 present in the C3 preparation as a result of storage, some inactivation occurs during chromatography. A proportion of the haemolytically inactive C3 has the same properties as C3 inactivated by freezing (C3u), namely uncleavable by convertase, cleavable by $\beta_1\text{H}$ and C3b inactivator, and supporting a very slow initial rate of factor B cleavage by factor D. The remainder of the haemolytically inactive C3 appears to support an initial rate of factor B cleavage more comparable with that seen with C3b and to be uncleavable by $\beta_1\text{H}$ and C3b inactivator. There may be several metastable forms of C3 with different conformations that retain one or more of the biological activities of the native molecule.

Since completion of the present study we have learnt that separation of C3, haemolytically inactive C3 and C3b has also been achieved, by using a different method, by Janatova *et al.* (1980). These authors also suggested the existence of multiple forms of C3.

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