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The Purification and Characterization of Bovine C4, the Fourth Component of Complement

By NUALA A. BOOTH, R. DUNCAN CAMPBELL and JOHN E. FOTHERGILL
Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, Scotland, U.K.

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The fourth component of complement, C4, was isolated from bovine plasma in high yield, by using simple purification techniques. The protein, like human component C4, is a β-globulin with a mol.wt. of about 200000 and consists of three polypeptide chains, α, β and γ, with apparent mol.wts. of 98000, 82000 and 32000 respectively. The chains of C4 have been separated by methods previously used for human C4. Their amino acid compositions are very similar to those of the human component, but differences in carbohydrate distribution have been observed. The haemolytic activity of bovine C4 is totally destroyed by incubation with bovine $C\bar{1}s$, the activated subcomponent of the first component of complement. Component C4, treated in this way, was shown to be cleaved in the α chain, which was decreased in mol.wt. by about 9000, corresponding to the removal of subcomponent C4a.

The classical pathway of complement activation (reviewed by Fothergill & Anderson, 1978) is initiated by the binding of C1, the first component of complement, to antibody–antigen complexes. Component C1 binds to the Fc region of IgG through its subcomponent C1q and this binding results in the sequential activation of C1r and C1s, the other two subcomponents of C1 (reviewed by Porter, 1977). Activated C1s acts as a proteinase and cleaves both C4 and C2 to produce two fragments from each; the larger ones, C4b and C2a, interact to become an active enzyme capable of continuing the activation process by cleaving C3.

Although C4b has no known enzymic activity, its role appears to be that of locating the C2a enzymic activity either by interaction with the cell surface (Müller-Eberhard & Lepow, 1965) or by attachment to immunoglobulin (Porter, 1977). Cleavage of C4 by $C\bar{1}s$ thus generates two specific binding sites on the large fragment C4b, one for C2 and another for cell surface or immunoglobulin. It also results in the cleavage of a small fragment, C4a, that has been reported to have some activity in contracting uterine muscle (Budzko & Müller-Eberhard, 1970).

Human C4 is a protein of mol.wt. 200000 with a three-chain structure. The approximate mol.wts. of the α, β and γ chains are 93000, 78000 and 33000 respectively (Schreiber & Müller-Eberhard, 1974; Gigli *et al.*, 1977; Bolotin *et al.*, 1977). The α chain is cleaved by activated C1 to produce C4a (Budzko & Müller-Eberhard, 1970; Patrick *et al.*, 1970).

Human C4 has a number of structural similarities to human C3 and C5, both of which consist of two chains, an α chain and a β chain of mol.wts. approx. 120000 and 75000 respectively (Nilsson *et al.*, 1972; Bokisch *et al.*, 1975; Molenaar *et al.*, 1975). Each is cleaved in the α chain to form a small polypeptide, C3a (Hugli, 1975) and C5a (Fernandez & Hugli, 1976) respectively. Both of these small fragments, known as anaphylatoxins, have potent biological activity (reviewed by Hugli, 1975). In the case of C3, the remainder of the molecule, C3b, is susceptible to further degradation of the α chain by C3b inactivator (Nagasawa & Stroud, 1977) and in this it resembles C4b (Shiraishi & Stroud, 1975). These common features have given rise to suggestions that components C4, C3 and C5 have evolved from a common ancestral protein (Schreiber & Müller-Eberhard, 1974). The similarities of C3a and C5a have already been described (Fernandez & Hugli, 1977).

In order to purify significant quantities of C4 for detailed structural studies, relatively large amounts of starting material are necessary. Bovine blood was therefore chosen. The aim of the work described in this paper was to purify C4 from bovine blood and to investigate its similarity to the human component by comparison of its structural and functional characteristics. This work was carried out in conjunction

Abbreviations used: 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. $C\bar{1}s$. SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; IgG, immunoglobulin G.

with a parallel study on the C1 components of bovine blood (Campbell *et al.*, 1979).

Materials and Methods

Materials

Materials were obtained from the following sources: sheep erythrocytes in Alsevers solution, Tissue Culture Services, Slough, Berks., U.K.; guinea-pig serum, Wellcome Reagents, Beckenham, Kent, U.K.; benzamidine hydrochloride hydrate, Aldrich Chemical Co., Gillingham, Dorset, U.K.; DEAE-Sephadex A-50, CM-Sephadex C-50, Sepharose 4B and Sephadex G-200, Pharmacia Fine Chemicals, Uppsala, Sweden; hydroxyapatite, Bio-Gel HTP, Bio-Rad Laboratories, Bromley, Kent, U.K.

C4-deficient guinea-pig serum was kindly provided by Professor P. J. Lachmann, M.R.C. Group on Mechanisms in Tumour Immunity, Cambridge.

Isolation of component C4

Bovine blood (9 vol.) was collected at the slaughterhouse directly into 1 vol. of 0.136M-trisodium citrate and transferred immediately into 1-litre centrifuge bottles containing 7.8g of benzamidine hydrochloride hydrate to give a final concentration of 50mM. The blood was chilled to 4°C as rapidly as possible. All further steps were carried out at 4°C unless stated otherwise. Conductivity and pH measurements were made at room temperature.

The blood was centrifuged at 1800*g* for 50min and the plasma pH was adjusted to approx. 5.6. The plasma (1.5 litres) was then diluted with 6 litres of 10mM-sodium acetate/1.4mM-acetic acid/2mM-EDTA/19mM-benzamidine, pH5.5, pre-cooled to 0°C. The pH was re-adjusted to 5.5 and the diluted plasma was stirred at 0°C for 2h, and then centrifuged at 1800*g* for 40min to remove the euglobulin precipitate. The supernatant fraction, the pseudoglobulin, was brought to 30% saturation with $(NH_4)_2SO_4$ at 0°C by slow addition of the solid and then stirred for 1h. The precipitate from centrifugation at 1800*g* for 30min was discarded and the supernatant brought to 50% saturation and centrifuged as before. The precipitate was retained and washed with 1 litre of 50%-saturated $(NH_4)_2SO_4$ in 28mM-sodium phosphate/2mM-EDTA/150mM-NaCl/10mM-benzamidine, pH6.6, 18.2mmho/cm and dialysed for 18h against 3×4 litres of this buffer. It is important to reach this stage of the preparation on the first day. The dialysed material was passed through a column (5cm×35cm) of DEAE-Sephadex A-50, equilibrated with 28.2mM-phosphate/2mM-EDTA/150mM-NaCl/10mM-benzamidine, pH6.6, at a flow rate of 100ml/h. The column was washed with 1.5 litres of the same buffer. It was then eluted with a linear gradient of 1 litre of 28mM-phosphate/2mM-EDTA/10mM-benzamidine, pH6.6, and 1 litre of 28mM-phosphate/2mM-EDTA/350mM-NaCl/10mM-benzamidine, pH6.6, followed by elution with 1 litre of the latter buffer. The fractions containing component C4 were pooled, concentrated by precipitation with 60%-saturated $(NH_4)_2SO_4$ and dialysed overnight against 3×700ml of 20mM-acetate/2.3mM-acetic acid/2mM-EDTA/150mM-NaCl/10mM-benzamidine, pH5.6. The protein was then passed through a column (2.5cm×8cm) of CM-Sephadex C-50, equilibrated with the same buffer. The column was washed with approx. 120ml of this buffer and then eluted with 20mM-acetate/2.3mM-acetic acid/2mM-EDTA/350mM-NaCl/10mM-benzamidine, pH5.6. The protein eluted by the latter buffer was precipitated with 60% saturated $(NH_4)_2SO_4$ and redissolved in approx. 10ml of 28mM-phosphate/2mM-EDTA/0.5M-NaCl/10mM-benzamidine, pH6.6. It was then applied to a column (2.5cm×10cm) of Sephadex G-200, equilibrated with the same buffer and eluted by upward flow at a rate of 12ml/h. The fractions containing C4 were pooled, concentrated and stored at 4°C.

Haemolytic assay of C4 activity

Veronal-buffered saline, *I* 0.065, pH7.3, containing 1mM-$MgCl_2$ and 0.15mM-$CaCl_2$ (VBS) was prepared by the method of Rapp & Borsos (1970). Samples of C4 (100μl) were prepared by serial dilution into VBS solution. To each was added 100μl of sheep erythrocytes sensitized with antibody (1.5×10^8 cells/ml) and 20μl of C4-deficient guinea-pig serum in a final volume of 1ml of VBS solution. After incubation at 37°C for 30min the suspension was centrifuged and the A_{412} of the supernatant was taken as a measure of haemolysis. Results are expressed in CH_{50} units, the reciprocal of the final dilution of test material which lyses 50% of the available cells.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Electrophoresis in polyacrylamide gel

Electrophoresis was performed in the presence of SDS in 5% polyacrylamide gels as described by Weber *et al.* (1972).

Preparation of antisera and of Sepharose–antiserum columns

Antisera were produced in rabbits as described previously (Campbell *et al.*, 1979). Before covalent linkage to Sepharose 4B, antiserum preparations were partially purified by precipitation with 40%-saturated $(NH_4)_2SO_4$. Sepharose 4B was activated with CNBr and coupled with the antiserum by the method of March *et al.* (1974).

The buffer used for the coupling procedure was 0.1M-$NaHCO_3$, pH8.3, containing 0.5M-NaCl. When

the reaction was carried out at 4°C for 20h, 6–8mg of protein was coupled per 1ml packed gel volume.

Immunological methods

Immunodiffusion and immunoelectrophoresis were performed as described previously (Campbell *et al.*, 1979). Quantitative immunoassay of component C4 was carried out by the method of Laurell (1966), using 2% (w/v) anti-C4 in 1% (w/v) agarose. A constant voltage gradient of 6V/cm was applied for 16h.

Treatment of component C4 with C1 esterase

Purified bovine C1s was activated by incubation with human $C\overline{1r}$ for 2h at 37°C. $C\overline{1r}$ activated by the method of Ziccardi & Cooper (1976) was added in two portions at 1h intervals to give a final ratio of C1r:C1s of 1:20 (w/w). C4 was incubated with $C\overline{1s}$ in a weight ratio of 100:1 for 30min at 37°C. The reaction was terminated by rapid cooling and the samples were desiccated before gel electrophoresis in the presence of SDS.

Separation of the polypeptide chains of component C4

The method described by Gigli *et al.* (1977) for the reduction and alkylation of human C4 and the separation of the polypeptide chains was used. A column of hydroxyapatite (1.6cm×35cm) was equilibrated with 10mM-sodium phosphate, pH6.4, containing 0.1% SDS and 0.2% NaN_3. Reduced and alkylated C4 (50mg) was loaded on the column in this buffer. The polypeptide chains were eluted with a gradient of 600ml of 0.2M-sodium phosphate, pH6.4, and 600ml of 0.5M-sodium phosphate, pH6.4, each containing 0.1% SDS and 0.2% NaN_3. The column eluate was monitored by absorbance at 280nm, and polyacrylamide-gel electrophoresis in the presence of SDS was performed on individual fractions before pooling tubes containing the separated chains. The polypeptide pools were dialysed exhaustively against 10mM-sodium phosphate, pH6.4, containing 0.2% NaN_3 and freeze-dried. SDS was removed when necessary by acid precipitation of the protein, followed by acetone washes (Bray & Brownlee, 1973).

Amino acid analyses

These were carried out as described by Campbell *et al.* (1979).

N-Terminal amino acid analysis

N-Terminal amino acids of the C4 polypeptide chains were dansylated by the method of Weiner *et al.* (1972), and the dansyl-amino acids were identified by t.l.c. on polyamide sheets. Before dansylation, the SDS and buffer salts were removed by the method of Bray & Brownlee (1973).

Results

Bovine C4 could be purified with a high yield of haemolytic activity by the procedures described (Table 1). A critical factor is the use of low pH throughout the purification, which resulted in much improved yields over those achieved at pH7.5, as reported earlier (Campbell *et al.*, 1977). The use of the proteolytic inhibitor benzamidine hydrochloride (Mares-Guia & Shaw, 1965) caused further increases in the yield of native C4. The precipitation of the euglobulin fraction and the $(NH_4)_2SO_4$ fractionation procedures were carried out strictly at 0°C to decrease proteolysis and the early steps were performed as rapidly as possible for the same reason.

Haemolytic assays on the plasma and pseudoglobulin fractions were unsatisfactory and recoveries from these stages were calculated from measurement of C4 by Laurell 'rockets' (Table 1). The pseudoglobulin contains all the plasma C4 and the recovery from the $(NH_4)_2SO_4$ fractionation is about 83%. Ion-exchange on DEAE-Sephadex A-50 (Fig. 1) gives a poor yield of component-C4 activity; no haemolytically inactive component C4 is found in the eluate when tested by immunodiffusion. The recoveries of C4 activity from CM-Sephadex C-50 and Sephadex G-200 columns are high. The protein which is not bound to CM-Sephadex C-50 in the presence of 150mM-NaCl consists mainly of IgG. Some component C4, as determined by reaction with anti-C4, remains associated with the IgG. However,

Table 1. *Purification of bovine C4 from 1.5 litres of plasma*

Assays of plasma and pseudoglobulin for C4 haemolytic activity were not reproducible and recovery of activity is therefore calculated with respect to the $(NH_4)_2SO_4$ fraction. Component C4 was determined by the Laurell 'rocket' procedure.

Purification step	Total protein (mg)	10^{-3}×Total CH_{50} units	Specific activity (CH_{50} units/mg)	Yield (% CH_{50} units)	Yield (% of C4)
Plasma	116000	—			100
Pseudoglobulin	107000	—			100
$(NH_4)_2SO_4$ fraction	39200	9425	240	100*	83.3
DEAE-Sephadex A-50 pool	446	2685	6020	28.4	19.7
CM-Sephadex C-50 pool	310	2175	7016	23.1	16.4
Sephadex G-200 pool	240	1872	7800	19.9	14.6

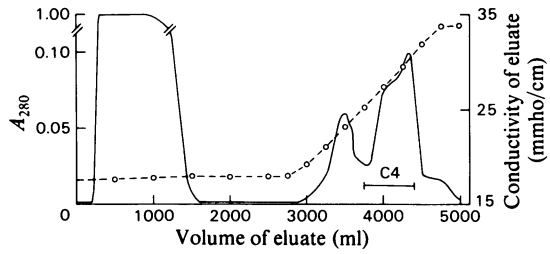

Fig. 1. *Chromatography of C4 on DEAE-Sephadex A-50*
The $(NH_4)_2SO_4$ fraction of the pseudoglobulin (725ml) was loaded on to a column (5cm×35cm) of DEAE-Sephadex A-50, equilibrated with 28mM-phosphate/2mM-EDTA/150mM-NaCl/10mM-benzamidine, pH6.6. After elution of the unbound protein, the salt concentration of the eluent was increased by use of a linear gradient to 350mM. ——, A_{280}; ○, conductivity of the eluate (mmho/cm).

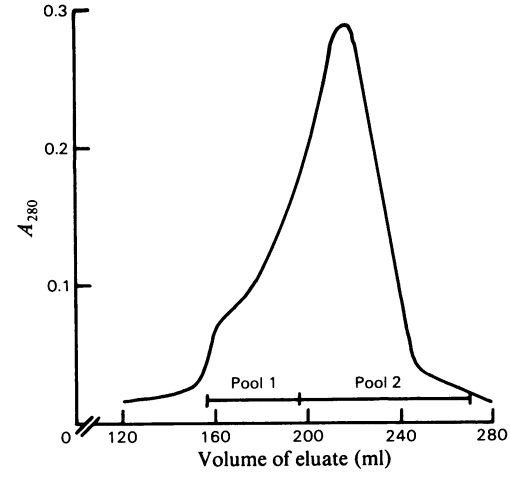

Fig. 2. *Gel filtration of component C4 on Sephadex G-200*
The C4-containing fractions from the CM-Sephadex C-50 column, pooled and concentrated, were loaded on to a Sephadex G-200 column (2.5cm×100cm) and eluted with 28mM-phosphate/2mM-EDTA/0.5M-NaCl/10mM-benzamidine, pH6.6. ——, A_{280}.

no activity is found in these fractions and it is likely that they contain C4b or some further degradation product of C4.

The final column in the procedure, Sephadex G-200, results in only a small degree of purification, but is useful because it partially resolves C4 from its major contaminant. The contaminant is eluted from the column slightly ahead of the bulk of the C4 (Fig. 2), but the peaks cannot be resolved completely, even after recycling. Two pools were taken from the eluate; pool 1 contains 85–90% contaminant, whereas pool 2 contains 90–95% C4. Component C4 was finally separated from the contaminant by a series of Sepharose-4B–antiserum columns. An antiserum was raised against the early preparation of C4 reported previously (Campbell *et al.*, 1977), which was found to consist mainly of C4b and which was contaminated with IgG. After absorption of the antiserum with commercial IgG, it was coupled to Sepharose 4B. A sample of pool 1 from the Sephadex G-200 eluate (Fig. 2) was passed through this column in 0.1M-phosphate buffer, pH7.0. The protein that was not retained was haemolytically inactive and gave a single band of apparent mol.wt. 200000 on polyacrylamide-gel electrophoresis in the presence of SDS, under reducing or non-reducing conditions. An antiserum was then raised to this purified contaminant and was coupled to Sepharose 4B. A column of this material is used as a final purification step where pure C4 is required. The component C4 eluted from this column contains no detectable impurities, except for traces of the polypeptides of apparent mol.wt. 42000 and 53000 reported by Gigli *et al.* (1977) to be partial degradation products of the α chain (Fig. 3*a*). For many experiments, including the separation of the polypeptide chains of C4, the C4 pool from Sephadex G-200 (Pool 2, Fig. 2), which contains 6–10% contaminant (Fig. 3*b*), was used without further purification.

Immunochemical characterization

Purified C4 gave a single precipitin line in Ouchterlony double diffusion against rabbit antiserum raised to earlier preparations of bovine C4, which consisted mainly of C4b. It gave no precipitin line against antisera raised in rabbits to bovine plasma, bovine IgG or bovine C3. A single precipitin arc in the β-globulin region was observed when the purified material was subjected to immunoelectrophoresis and reacted with anti-bovine C4b. Bovine C4 did not cross-react with rabbit antiserum raised to human C4.

Haemolytic activity of bovine C4

Bovine C4 was found to be compatible with guinea-pig complement components and could be assayed simply by using C4-deficient guinea-pig serum. It is noteworthy that, although bovine C1 is compatible with guinea-pig C2–9 (Campbell *et al.*, 1979), bovine C4 could not be assayed by using bovine C1, guinea-pig C2 and guinea-pig C3–9.

The haemolytic activity of bovine C4 was stable to heating at 56°C for 30min, as is C4 from human and guinea-pig serum (Bier *et al.*, 1945). However, it was stable to concentrations of ammonium ions which result in the loss of activity of guinea-pig C4 (Gordon *et al.*, 1926).

Treatment of C4 with C1 esterase

Incubation of purified bovine C4 with bovine $C\bar{1}s$ for 30min at 37°C resulted in complete loss of C4 haemolytic activity. This treatment also caused a decrease in the apparent mol.wt. of a large proportion of the α chain from 98000 to about 89000, with the production of a peptide of approximate mol.wt. 9000 (Fig. 3*c*). This peptide, C4a, has been purified and characterized.

Chain structure of bovine component C4

Purified C4 has an apparent mol.wt. of 200000, as estimated from polyacrylamide-gel electrophoresis in the presence of SDS, by comparison with standard proteins. On reduction, three bands of apparent mol.wt. 98000, 82000 and 32000 appear (Fig. 3*a*). From scans of the Coomassie Blue-stained gels at 560nm, the ratio of the bands was calculated as 2.7:2:1.0 and the ratio of the molecular weights of the chains is 3.1:2.6:1.0. These results are compatible with a three-chain structure for bovine C4, in agreement with the structure proposed for human C4 (Schreiber & Müller-Eberhard, 1974; Gigli *et al.*, 1977; Bolotin *et al.*, 1977).

Separation of the polypeptide chains of component C4

The polypeptide chains of bovine C4 were separated by the method used for the chains of human C4 (Gigli *et al.*, 1977). A gradient of sodium phosphate eluted the γ, β and α chains; the pools obtained were at phosphate concentrations of 0.31–0.33M, 0.35–0.37M and 0.39–0.42M respectively. The procedure yielded 190nmol of γ chain and about 110nmol each of β and α chains from 250nmol of reduced carboxymethylated C4. The yields of the α and β chains are low owing to incomplete resolution, but can be increased by repeated chromatography. The isolated chains showed only traces of impurities (Figs. 3*d*–3*f*). The inclusion of 0.2% NaN_3 in all buffers was necessary to inhibit bacterial contamination and consequent degradation of the polypeptide chains.

Amino acid analyses

The amino acid compositions of component C4 and of its polypeptide chains are shown in Table 2. There are few striking differences between the composition of the individual chains, but the high cysteine content of the γ chain is notable.

N-Terminal amino acid analyses

The *N*-terminal amino acids of the three chains, as determined by the dansyl method, are: α chain, asparagine or aspartic acid; β chain, lysine; γ chain, glutamine or glutamic acid.

Carbohydrate content

On electrophoresis of component C4 under reducing conditions on polyacrylamide gels in the presence of SDS, followed by staining for carbohydrate by the periodic acid/Schiff method, only two bands were observed. These bands corresponded to apparent mol.wts. of 98000 for the lighter stained band and 82000 for the heavier stained band. No band was visible corresponding to 32000 mol.wt., even on

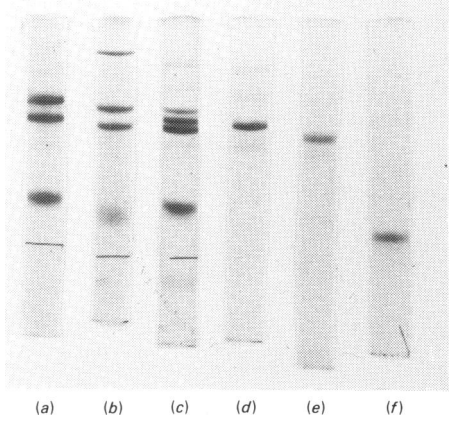

Fig. 3. *SDS/polyacrylamide-gel electrophoresis*
Electrophoresis of component C4 and its peptide chains on 5% polyacrylamide gel in the presence of SDS. Gels were run under reducing conditions. (*a*) Purified C4; (*b*) C4 plus contaminating protein (pool 2, Fig. 2); (*c*) C4 after treatment with $C\bar{1}s$; (*d*) α chain of C4; (*e*) β chain of C4; (*f*) γ chain of C4.

Table 2. *Amino acid composition of bovine C4 and its three polypeptide chains*
N.D., Not determined

	Amino acid compositions (residues/100 residues)			
	C4	α chain	β chain	γ chain
Cys	1.2	1.4	1.1	2.7
Asp	8.2	8.2	8.2	7.9
Thr	4.8	5.5	4.4	5.3
Ser	8.2	7.1	9.5	7.6
Glu	11.3	13.1	11.3	13.4
Pro	4.8	5.6	5.7	5.0
Gly	8.4	8.0	8.6	8.0
Ala	7.5	9.0	6.6	8.2
Val	9.0	7.7	9.6	7.4
Met	2.0	2.2	1.5	1.3
Ile	3.5	3.7	4.3	3.0
Leu	10.7	11.3	9.9	9.4
Tyr	2.8	2.4	2.1	3.6
Phe	4.2	3.5	4.5	3.8
His	2.2	2.1	1.8	1.7
Lys	4.8	3.9	5.1	4.5
Arg	6.0	5.2	5.8	7.1
Trp	0.4	N.D.	N.D.	N.D.

heavily loaded gels. These results suggest that only the α and β chains of component C4 contain carbohydrate and that the β chain has a higher content than the α chain.

Discussion

A detailed structural study of component C4 necessitates the purification of large amounts of protein. The procedure described here yields 120–150mg of intact, haemolytically active C4/litre of bovine plasma. Yields of component-C4 activity were high at every stage except the DEAE-Sephadex column fractionation. Failure to detect haemolytically inactive component C4 in the column eluate by immunodiffusion suggests that the remaining material is probably bound to the column. Even so, the overall yield of 15% is relatively high for a haemolytically active complement component.

The purified material contains up to 10% of a contaminating protein which was not removed by conventional purification techniques, but which could be removed by passage through a column of antiserum raised to the contaminant and covalently bound to Sepharose. The contaminant has the same apparent molecular weight as unreduced C4 on polyacrylamide-gel electrophoresis in the presence of SDS and initially was thought to be a single-chain form of C4, as reported by Hall & Colten (1977), who found it was synthesized in guinea-pig liver homogenate, and Gigli (1978), who isolated it from human plasma and serum. However, its failure to interact with antiserum to C4 suggests that it is not this precursor molecule, but another protein that is readily degraded by blood proteinases and is not normally found when steps are not taken to minimize proteolysis.

Careful handling of the blood in the early stages of the purification and the addition of the proteolytic inhibitor benzamidine hydrochloride, are crucial in obtaining good yields of active C4. This has also been the experience of workers purifying human C4, where di-isopropyl phosphorofluoridate (Gigli *et al.*, 1977) and phenylmethylsulphonyl fluoride and ε-aminohexanoic acid (Bolotin *et al.*, 1977) have been used.

This study on bovine C4 was greatly facilitated by the simple assay system using C4-deficient guinea-pig serum, which has also been used to assay C4 in human, guinea-pig and monkey serum (Gaither *et al.*, 1974). Bovine C1 has also been found to be compatible with the complement components of guinea-pig serum (Campbell *et al.*, 1979). However, we find that bovine C4 cannot be assayed by using bovine C1, guinea-pig C2 and guinea-pig C3–9, which indicates some limit to the compatibility of the early bovine and guinea-pig complement components. Moreover, in the assays of bovine C4 and of bovine C1 (Campbell *et al.*, 1979), difficulties were noted in achieving quantitative haemolysis in the crude fractions of the preparations. These observations perhaps help to explain early reports that bovine serum was non-haemolytic in standard assays and that components C4 and C2, in particular, were not detectable (Rice & Crowson, 1950).

Bovine C4 is a β-glycoprotein of mol.wt. about 200000 and has a three-chain structure, with apparent mol.wts. for the α, β and γ chains of 98000, 82000 and 32000 respectively. These values are in good agreement with those reported for human C4 (Schreiber & Müller-Eberhard, 1974; Gigli *et al.*, 1977; Bolotin *et al.*, 1977).

Both the α and β chains of bovine C4 contain carbohydrate, the β chain having a higher content. No carbohydrate was detected in association with the γ chain. This differs from the result obtained with human C4, where all three chains contain carbohydrate (Schreiber & Müller-Eberhard, 1974). Detailed carbohydrate analysis on the separated chains of human C4 showed that the α and β chains contained 8.6% and 5.6% carbohydrate respectively and that the γ chain had a low carbohydrate content (Gigli *et al.*, 1977).

The polypeptide chains of component C4 were separable by the method developed for those of human C4 (Gigli *et al.*, 1977) and amino acid compositions were obtained for each of the chains. The results are in broad agreement with those obtained for human C4. The compositions of bovine and

Table 3. *Comparison of amino acid compositions of human and bovine C4*

	Amino acid composition (residues/100 residues)			
	Human C4 (Budzko & Müller-Eberhard, 1970)	Human C4 (Gigli *et al.*, 1977)	Human C4 (Bolotin *et al.*, 1977)	Bovine C4
Cys	2.7	1.5	1.7	1.2
Asp	7.7	8.1	7.9	8.2
Thr	6.0	5.3	5.2	4.8
Ser	8.6	8.2	8.1	8.2
Glu	10.0	11.9	11.4	11.3
Pro	5.4	5.6	5.6	4.8
Gly	8.0	7.5	7.3	8.4
Ala	6.9	7.9	8.0	7.5
Val	7.6	7.7	7.7	9.0
Met	1.5	1.5	1.6	2.0
Ile	3.3	3.1	3.2	3.5
Leu	11.6	11.6	11.7	10.7
Tyr	2.8	3.3	3.0	2.8
Phe	4.4	3.8	3.7	4.2
His	2.6	2.4	2.3	2.2
Lys	5.6	4.5	4.4	4.8
Arg	5.2	5.4	6.1	6.0
Trp	N.D.	1.2	1.1	0.4

human C4 are compared in Table 3 and all the most notable features, such as the high content of glutamic acid and leucine, are common to both proteins.

The *N*-terminal amino acids of the chains were identified as: α chain, aspartic acid or asparagine; β chain, lysine; γ chain, glutamic acid or glutamine. These results are in exact agreement with those obtained for the human chains (Bolotin *et al.*, 1977; Gigli *et al.*, 1977), though in the latter study analysis of phenylthiohydantoins allowed their unambiguous identification as asparagine, lysine and glutamic acid respectively.

Incubation of bovine C4 with bovine $C\bar{1}s$ results in total loss of haemolytic activity, as previously shown for human C4 (Müller-Eberhard & Lepow, 1965). This effect is associated with cleavage of the α chain into an α' chain of about 89000 mol.wt. and a small peptide C4a. These two fragments are not covalently linked, but it is not known whether they normally separate under physiological conditions. Cleavage of the α chain also occurs on activation of human C4, though there have been some differences of opinion about the size of the C4a peptide (Budzko & Müller-Eberhard, 1970; Patrick *et al.*, 1970; Schreiber & Müller-Eberhard, 1974).

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