The Binding of Human Complement Component C4 to Antibody–Antigen Aggregates

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The binding of human complement component C4 to antibody–antigen aggregates and the nature of the interaction have been investigated. When antibody–antigen aggregates with optimal C1 bound are incubated with C4, the C4 is rapidly cleaved to C4b, but only a small fraction (1–2%) is bound to the aggregates, the rest remaining in the fluid phase as inactive C4b. It has been found that C4b and the antibody form a very stable complex, due probably to the formation of a covalent bond. On reduction of the C4b–immunoglobulin G (IgG) complex, the β and γ chains, but not the α' chain, of C4b are released together with all the light chain, but only about half of the heavy chain of IgG. The reduced aggregates contain two main higher-molecular-weight complexes, one shown by the use of radioactive components to contain both IgG and C4b and probably therefore the α' chain of C4b and the heavy chain of IgG, and the other only C4b and probably an α' chain dimer. The aggregates with bound C1 and C4b show maximal C3 convertase activity, in the presence of excess C2, when the α' -H chain component is in relatively highest amounts. When C4 is incubated with C1s in the absence of aggregates, up to 15% of a C4b dimer is formed, which on reduction gives an α' chain complex, probably a dimer. The apparent covalent interaction between C4b and IgG and between C4b and other C4b molecules cannot be inhibited by iodoacetamide and hence cannot be catalysed by transglutaminase (factor XIII). The reaction is, however, inhibited by cadaverine and putrescine and ^{14}C -labelled putrescine is incorporated into C4, again by a strong, probably covalent, bond. It is suggested that a reactive group, possibly an acyl group, is generated when C4 is activated by C1 and that this reactive group can react with IgG, with another C4 molecule, or with water.

The activation of the classical pathway of complement by immune complexes is initiated by the binding of the first component (C1) through the C1q subcomponent to the Fc region of the antibody molecule. Binding of C1q results in the sequential activation of C1r and C1s, the other two subcomponents of the C1 complex, and the development of proteolytic activity in C1s, which is responsible for the activation of C4 and C2 (Porter

& Reid, 1978). Cleavage of C4 by C1s results in the generation of two specific binding sites in activated C4. One is unstable and allows the molecule to bind to antibody–antigen aggregates and to acceptor sites on the cell surface (Müller-Eberhard & Lepow, 1965; Cooper & Müller-Eberhard, 1968; Goers & Porter, 1978). The other binding site is stable and forms the acceptor site for C2 (Müller-Eberhard *et al.*, 1967; Kerr, 1980). C2 is then cleaved by C1s to form the complex proteinase C4 $\bar{2}$ (C3 convertase) (Müller-Eberhard *et al.*, 1967; Polley & Müller-Eberhard, 1968; Kerr, 1980), which activates C3. Some of the activated C3 can be associated with the C4 $\bar{2}$ complex, to give C4 $\bar{2}$ 3, which changes its specificity from C3 activation to C5 activation (Cooper & Müller-Eberhard, 1970; Goldlust *et al.*, 1974).

The binding site for activated C4 on antibody–antigen aggregates or antibody-coated red cells, and

Abbreviations used: the nomenclature of complement components and subcomponents is that recommended by the World Health Organisation (1968). Activated components are indicated by a bar, e.g. C1s. iPr $_2$ P-F, diisopropyl phosphorofluoridate; IgG, immunoglobulin gamma; Fab, N-terminal half of the heavy chain and light chain; Fc, C-terminal half of the heavy chain dimer; (Fab') $_2$, N-terminal half of the heavy chain and light chain from peptic digestion of IgG; Fd, N-terminal half of the heavy chain; SDS, sodium dodecyl sulphate.

the nature of the binding interaction, are far from clear. C4 is rapidly activated by C1, but only a small fraction (2–8%) is bound, the rest remaining in the fluid phase as inactivated C4b (Cooper & Müller-Eberhard, 1968; Goers & Porter, 1978). Binding of C4 to antibody-coated cells is 10–13 times greater than to aggregates, but the number of effective C4 molecules, as judged by their capacity to form C3 convertase in the presence of C2, is approximately the same (Goers & Porter, 1978). This suggests that effective C4 molecules may be those that are bound to the antibody. Goers & Porter (1978) have suggested that binding of activated C4 to antibody–antigen aggregates may occur via the Fab arm of the antibody molecule.

The present paper describes the further characterization of the binding site for activated C4 on antibody–antigen aggregates. The results suggest that activated C4 does not bind to the C1 subcomponents, but appears to bind to the antibody molecule. This interaction takes place between the α' chain of C4b and the Fd portion of the heavy chain of IgG. Characterization of the binding interaction between C4b and IgG suggests that the bond may be covalent in nature and may be similar to that described for C3 binding to cell surfaces (Law & Levine, 1977; Law *et al.*, 1979).

Materials and Methods

Materials

Chemicals were obtained as follows: iPr_2P -F, Calbiochem, San Diego, CA, U.S.A.; SDS and H_2O_2 (Aristar), BDH Chemicals, Poole, Dorset, U.K.; Coomassie Blue R-250, Bio-Rad, Richmond, CA, U.S.A.; dimethyl suberimidate dihydrochloride, Pierce & Warriner, Chester, U.K.; DEAE-Sephadex A-50, DEAE-Sepharose CL-6B, Sepharose 6B and Sephadex G-50, Pharmacia Fine Chemicals, Uppsala, Sweden; $Na^{125}I$ (100 mCi/ml) and $[1,4-^{14}C]$ putrescine dihydrochloride (116 Ci/mol), The Radiochemical Centre, Amersham, Bucks., U.K.; chloramine-T, putrescine dihydrochloride, cadaverine dihydrochloride, catalase and lactoperoxidase, Sigma Chemical Co., Poole, Dorset, U.K.; Industrex C X-ray film, Kodak, London, U.K.; salicylhydroxamic acid, Aldrich Chemical Co., Gillingham, Dorset, U.K.

Outdated human plasma was obtained from the Churchill Hospital, Oxford, U.K. It was made 20 mM with $CaCl_2$ and left to clot overnight at 4°C. The clot was removed by centrifugation and the serum was stored at –20°C.

Complement components

Functionally pure human C1 was obtained as a euglobulin precipitate from human serum as described in Gigli *et al.* (1976). Fractions were stored at

–70°C and appeared to be stable for months. Before adsorption on antibody–antigen aggregates the C1 was diluted with 1 vol. of 20 mM Tris/HCl, pH 7.4, and the pH was adjusted to 7.4 with 1 M-NaOH.

Human C1q, C1r and C1s were prepared as described in Reid (1974) and Gigli *et al.* (1976).

Human C4 was prepared by a modification of the method of Gigli *et al.* (1977). Following euglobulin precipitation of 1 litre of serum at pH 7.4 and pH 5.5 the pseudoglobulin was adjusted to pH 8.6 and the ionic strength was raised to 0.185 M by addition of NaCl. The solution was made 5 mM in iPr_2P -F, then applied to a column (7 cm $\times$ 25 cm) of DEAE-Sephadex A-50 equilibrated at 4°C in 0.185 M-NaCl/0.02% NaN_3 /0.01 M-Tris, pH 8.6. The column was washed with the starting buffer until the A_{280} of the eluate had fallen to below 0.04. Then a linear gradient composed of 2.5 litres of 0.185 M-NaCl/0.02% NaN_3 /0.01 M-Tris/HCl, pH 8.6, and 2.5 litres of 0.25 M-NaCl/0.02% NaN_3 /0.01 M-Tris/HCl, pH 8.6 was applied. Elution with the latter buffer was continued until no further protein was eluted from the column. The fractions containing C4 were pooled, concentrated to 40 ml by ultrafiltration and diluted with ice-cold distilled water to lower the NaCl concentration to 0.195 M. The sample was made 1 mM in iPr_2P -F, then applied to a column (5 cm $\times$ 35 cm) of DEAE-Sepharose CL-6B equilibrated at 4°C in 0.2 M-NaCl/0.02% NaN_3 /0.01 M-Tris/HCl, pH 8.6. The flow rate of the column was 50 ml/h. The column was eluted with starting buffer, C4 was retarded on the column and was separated from contaminating protein which eluted from the column ahead of C4. The fractions containing C4 were pooled, concentrated by ultrafiltration to about 25 ml, then stored at 2°C after being made 1 mM in iPr_2P -F. This material was functionally and immunochemically free from C1, C2 and C3 and was used for titration experiments. The concentration of C4 in the pool was determined by the specific radioimmunoassay for C4 described by Goers & Porter (1978). Purified C4 for iodination, immunization and standards in the specific radioimmunoassay was obtained by passing the C4 pool from the DEAE-Sepharose CL-6B column over a column of Sepharose 6B (Gigli *et al.*, 1977).

Human C2 was prepared by a modification of the procedure of Kerr & Porter (1978) as described by Kerr (1979).

Human C3 was prepared by a modification of the procedure of Tack & Prahl (1976) as described by Kerr (1980).

β 1H was a gift from Dr. R. B. Sim of this department.

Haemolytic assays of complement components

Glucose (dextrose)/saline/veronal/gelatin buffer and saline/veronal/gelatin buffer containing 0.04 M-

EDTA were prepared as described by Nelson *et al.* (1966). The haemolytic activity of whole serum complement CH₅₀, C1, C4, C2 and C3 were determined by the methods of Mayer (1961), Borsos & Rapp (1967), Gigli *et al.* (1977), Kerr & Porter (1978) and Kerr (1980) respectively.

Antibody preparations

Antisera to human C1q, C1s, C4 and ovalbumin were prepared as described in Goers & Porter (1978).

Antibody-antigen aggregates

Ovalbumin-anti-ovalbumin aggregates were formed with rabbit anti-ovalbumin IgG. The IgG fraction was obtained as described by Wilkinson (1969). (Fab')₂ fractions of whole rabbit antibody were prepared by pepsin digestion (Fanger *et al.*, 1970). Ovalbumin-anti-ovalbumin aggregates were prepared at equivalence as described in Gigli *et al.* (1976). Mixed antibody/(Fab')₂ aggregates containing 80% (Fab')₂ and 20% IgG (molar ratios) were prepared with optimal ovalbumin concentrations at 4°C with constant stirring.

To prepare antibody-antigen aggregates with C1 bound, samples of aggregates in 100 mM-NaCl/2 mM-CaCl₂/10 mM-Tris/HCl, pH 7.4, were incubated with an appropriate volume of the dilute euglobulin at 30°C for 30 min. The aggregates were centrifuged and washed three times with the NaCl/CaCl₂/Tris buffer, pH 7.4. The antibody-antigen aggregates with C1 bound were then used for titration experiments.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide slab gels of various acrylamide concentrations were prepared by the method of Laemmli (1970). SDS/polyacrylamide disc gel electrophoresis was carried out as described by Weber *et al.* (1972). Preparation of samples for electrophoresis was as described in Dodds *et al.* (1978). After electrophoresis the gels were stained for protein with Coomassie Blue. Slab gels were dried down and radioautography was carried out by exposing the dried gel to a Kodak Industrex C film. Radioautographs were developed using Kodak DX-80 developer and Kodak FX-40 fixer.

To calibrate gels for molecular weight estimations under reducing conditions the following protein markers were used: β1H (mol.wt. 150 000), the α and β chains of C3 (mol.wts. 115 000 and 75 000 respectively), phosphorylase a (mol.wt. 94 000), bovine serum albumin (mol.wt. 68 000) and ovalbumin (mol.wt. 44 500). Ovalbumin cross-linked with dimethyl suberimidate by the method of Carpenter & Harrington (1972) was also used as a molecular weight marker. Gels run under non-

reducing conditions were calibrated using cross-linked ovalbumin.

Radio-iodination procedures

Radio-iodination of anti-ovalbumin IgG fractions with ¹²⁵I was done with chloramine-T (Byrt & Ada, 1969). To 2 mg of IgG in 1 ml of 150 mM-NaCl/10 mM-phosphate, pH 7.4, was added 1 mCi of carrier-free ¹²⁵I⁻, 100 μl of dimethyl sulphoxide and 50 μl of chloramine-T (2 mg/ml). After 3–4 min on ice, 400 μl of sodium metabisulphite (5 mg/ml) was added to stop the reaction. Free iodide was separated on a column (1 cm × 25 cm) of Sephadex G-50 equilibrated with 0.15 M-NaCl/0.01 M-phosphate, pH 7.4. IgG was labelled to 0.02 g-atom of ¹²⁵I/mol of IgG. Anti-ovalbumin (Fab')₂ preparations were labelled with ¹²⁵I by the same procedure, except that the sodium metabisulphite was omitted and 400 μl of a saturated tyrosine solution was added to stop protein iodination. (Fab')₂ was labelled to 0.015 g-atom of ¹²⁵I/mol of (Fab')₂.

Radio-iodination of antibody preparations for the radioimmunoassay was carried out as described in Goers & Porter (1978).

Radio-iodination of C4 with ¹²⁵I was carried out by a modification of the method of Reboul *et al.* (1979), using lactoperoxidase. To 300–400 μg of C4 in 150 mM-NaCl/5 mM-EDTA/5 mM-iPr₂P-F/50 mM-Tris/HCl, pH 7.4, was added 1 mCi of carrier-free ¹²⁵I⁻, 50 μl of lactoperoxidase (1 mg/ml; made 10 mM in iPr₂P-F before use) and 20 μl of H₂O₂ (1:20 000 dilution). The reaction was carried out at 4°C. At times 10, 20 and 30 min 20 μl of the 1:20 000 diluted H₂O₂ was added. Ten min after the final addition of H₂O₂ the reaction was terminated with 50 μl of 10% (w/v) NaN₃. Catalase (100 μl of 25 mg/ml) was added and free ¹²⁵I⁻ was separated by gel filtration on a column (1 cm × 25 cm) of Sephadex G-50 equilibrated in 150 mM-NaCl/0.02% NaN₃/0.05 M-Tris/HCl, pH 7.4. The average incorporation of ¹²⁵I into C4 was 0.02–0.08 g-atom of ¹²⁵I/mol of C4. This labelling procedure appeared to have very little effect on the haemolytic activity of C4, or on the ability of C1s to cleave and activate C4 in solution. The distribution of radioactivity in C4 was determined on SDS/8.5% (w/v) polyacrylamide slab gels after reduction and alkylation and was as follows: approximately 35% of the label was in the α chain, 34% of the label was in the β chain and 31% of the label was in the γ chain. For titration experiments the ¹²⁵I-labelled C4 was diluted with cold C4 such that approximately the same specific radioactivity of ¹²⁵I-labelled C4 was used in each experiment. Samples of antibody-antigen aggregates with C1 bound were incubated with ¹²⁵I-labelled C4 in 0.1 M-NaCl/2 mM-CaCl₂/10 mM-Tris/HCl, pH 7.4, at 30°C for 30 min. After centrifugation and washing three times with the NaCl/

CaCl₂/Tris buffer, pH 7.4, the aggregates were transferred to fresh tubes and samples were taken for counting to determine the bound C4.

Samples containing ¹²⁵I were counted for radioactivity in an LKB 1270 Rackgamma γ -radiation counter. Gels to be counted for radioactivity were sliced into 2 mm segments.

Radioimmunoassay for C4

The specific radioimmunoassay for C4 was carried out as described by Goers & Porter (1978).

Liquid scintillation counting

Samples for liquid scintillation counting were added to 10 ml of 1,4-dioxan containing 0.5% (w/v) 2,5-diphenyloxazole and 2% (w/v) naphthalene and counted in an LKB 1210 Ultrabeta liquid-scintillation counter.

Results

Optimal conditions for maximum C4 binding and development of maximum C3 convertase activity in the presence of excess C2 on antibody-antigen aggregates

Antibody-antigen aggregates with increasing amounts of C1 bound were incubated at 30°C for 30 min with constant amounts of ¹²⁵I-labelled C4 in 100 mM-NaCl/2 mM-CaCl₂/10 mM-Tris/HCl, pH 7.4. After centrifugation and thorough washing of the aggregates with the same buffer, samples were taken for counting to determine the bound C4. Maximum C4 binding occurred when 24 250 units of C1 were added to 50 μ g of the antibody-antigen aggregates (Table 1). On electrophoresis of samples on SDS/10% (w/v) polyacrylamide gels the α' chain of C4b was barely detectable after reduction at this amount of C1, but bands with a mobility similar to unreduced C4 were apparent (Fig. 1). As the C4 β

Table 1. *Binding of C4 to IgG and relative C3 convertase formation with increasing amounts of C1 bound to antibody-antigen aggregates*

Samples (50 μ g) of antibody-antigen aggregates with increasing amounts of C1 bound were incubated at 30°C for 30 min in 100 mM-NaCl/2 mM-CaCl₂/10 mM-Tris/HCl, pH 7.4, with 5000 molecules of ¹²⁵I-labelled C4/1000 molecules of IgG. After centrifugation at 2000 *g* for 15 min the aggregates were washed three times with the NaCl/CaCl₂/Tris/HCl, pH 7.4, buffer and samples were taken for counting to determine the bound C4. Relative C3 convertase activity was measured as the reciprocal of the time to inactivate 50% of the added C3 in the presence of excess C2 and is expressed as relative to Expt. 2 = 100. Samples of the aggregates were also washed twice with 100 mM-NaCl/5 mM-EDTA/10 mM-Tris/HCl, pH 7.4, and the amount of C4 in the supernatants was determined by counting.

Expt.	C1 added (units)	C4 bound (molecules/1000 molecules of IgG)	Relative C3 convertase activity	C4 removed by EDTA washes (molecules/1000 molecules of IgG)
1	4850	75	50	11
2	24 250	110	100	20
3	121 250	95	25	34
4	242 500	65	<15	34

Fig. 1. *Radioautograph of SDS/10% (w/v) polyacrylamide slab gels showing binding of ¹²⁵I-labelled C4b to antibody-antigen aggregates carrying increasing amounts of C1*

Radioautograph of SDS/10% (w/v) polyacrylamide slab gel run by the method of Laemmli (1970) showing ¹²⁵I-labelled C4b bound to antibody-antigen aggregates carrying increasing amount of C1. (a) Aggregates before the EDTA wash; (b) EDTA wash; (c) aggregates after the EDTA wash. Unreduced samples are on the left and reduced samples on the right of each pair. The numbers underneath each sample correspond to the numbered samples in Table 1.

and γ chains showed no change in intensity, these higher molecular weight bands most probably come from aggregated α' chains. At higher amounts of C1 on the aggregates, the α' chain of C4b became visible, but this could be removed if the aggregates were washed further with EDTA-containing buffers (Fig. 1), which also removed C1r and C1s. The maximum C3 convertase activity after addition of excess C2 also occurred when 50 μ g of antibody-antigen aggregates were offered 24250 units of C1 (Table 1). This was lost after the EDTA buffer wash, but was restored if C1r and C1s were added back. The C3 convertase activity appears therefore to be associated with the strongly-bound C4b, containing no monomer α' chains but only higher molecular weight aggregates of the α' chain. For maximum C4 binding and maximum C3 convertase activity an optimal amount of C1 had to be added corresponding to approx. 485 units/ μ g of antibody-antigen aggregates, and this amount was used in subsequent experiments.

Ability of antibody-antigen aggregates with optimal C1 bound to bind C4

Antibody-antigen aggregates with optimal C1 bound were incubated with increasing concentrations of 125 I-labelled C4 at 30°C for 30 min in 0.1 M-NaCl/0.002 M-CaCl₂/0.01 M-Tris/HCl, pH 7.4. After incubation, the aggregates were centrifuged and washed and samples were taken for counting to determine the bound C4. With increasing C4 concentration, C4b binding to the aggregates increased and a maximum of about 340 molecules of C4 were bound per 1000 molecules of antibody (Fig. 2). Maximum C3 convertase activity, developed in the presence of excess C2, occurred when only 40%–50% of the maximum C4 was bound (Fig. 2). These values are similar to those reported previously (Goers & Porter, 1978) and again the bound C4 represents only 1–2% of the C4 added.

Electrophoresis of the aggregates in polyacrylamide gels using SDS-containing buffers showed increasing amounts of the C4 β and γ chains as the C4 binding was increased, but no α' chain (Fig. 3). Higher molecular weight bands increased proportionately, and were presumably derived from the α' chain. If 125 I-labelled antibody-antigen aggregates with unlabelled C4b bound were run on SDS/8.5% (w/v) polyacrylamide-gel slabs and the gel was sliced into 2 mm segments, the radioactivity showed that the major higher molecular weight band containing the α' chain (α' -H) also contained radioactivity due to IgG (Fig. 4). The second major α' chain band (α' - α') however, contained no labelled IgG. This suggests that the α' chain can form a bond resistant to reduction and boiling in SDS/urea-containing buffers, both with the antibody and with other C4b molecules. Optimum C3

Fig. 2. Binding of C4 and development of C3 convertase activity on antibody-antigen aggregates carrying optimal amounts of C1

Samples (40 μ g) of antibody-antigen aggregates carrying optimal amounts of C1 were incubated with increasing concentrations of 125 I-labelled C4 in 100 mM-NaCl/2 mM-CaCl₂/10 mM-Tris/HCl, pH 7.4, at 30°C for 30 min. After incubation, the aggregates were centrifuged at 2000g for 15 min, washed three times with the NaCl/CaCl₂/Tris/HCl, pH 7.4, buffer and transferred to fresh tubes. Samples were then taken for counting to determine the bound C4. The ability of the aggregates to form C3 convertase was determined by incubating samples with 1000 units of C2 and 150 units of C3 at 30°C for 30 min. The aggregates were then centrifuged at 2000g for 5 min and the residual C3 activity in the supernatant was determined by haemolytic assay. The percentage of C3 destroyed (●) and of C4 bound (O) is plotted versus the amount of C4 added to the samples.

convertase activity is reached when the α' -H band is at a maximum amount relative to other bands (positions 4 and 5, Fig. 3) suggesting that formation of the C3 convertase enzyme is dependent on a strong, perhaps covalent, interaction between C4b and IgG. The stability of the C4b-IgG interaction is emphasized by the absence of significant displacement of 125 I-labelled C4b when the aggregates are incubated at 30°C for 1 h in the presence of a several-thousand-fold excess of unlabelled C4.

If antibody-antigen aggregates with no bound C1 attached, but with equivalent amounts of soluble C1s present, are incubated with increasing amounts of C4, very much less C4 is bound (Fig. 5) showing that only C1s bound to the antibody through C1q and C1r can activate C4 in such a way that it forms a strong bond with the antibody, although the C1s in solution rapidly converts the C4 to C4b. On electrophoresis in SDS/polyacrylamide gels of the C4-C1s incubation mixture no α' -H band is visible, but the α' - α' band is visible. The α' - α' band is

Fig. 3. Electrophoresis in SDS/8.5% (w/v) polyacrylamide-slab gel of antibody-antigen aggregates with increasing amounts of C4 bound

(a) Samples of antibody-antigen aggregates with increasing amounts of C4 bound, from points shown in Fig. 2, were run under reducing conditions on an SDS/8.5% (w/v) polyacrylamide-slab gel by the method of Laemmli (1970). Proteins were stained with Coomassie Blue. (b) Radioautograph of the gel. Radioautographs of C4 and C4b are shown for comparison.

Fig. 4. SDS/polyacrylamide gel electrophoresis pattern of ^{125}I -labelled antibody-antigen aggregates with C4 bound

A sample of ^{125}I -labelled antibody-antigen aggregates with C4 bound was electrophoresed under reducing conditions in an SDS/8.5% (w/v) polyacrylamide gel (Laemmli, 1970). After electrophoresis the gel was sliced into 2 mm segments which were counted for radioactivity. The relative positions of the α' - α' dimer and β chain of C4b are given for comparison.

apparent when C4 and C $\bar{1}$ s are incubated alone in the absence of aggregates and can increase to about 15% of the total α' chain of the C4b formed. This suggests that when C4 is hydrolysed by C $\bar{1}$ s a reactive group in the α' chain forms a strong bond, preferentially with IgG if the C $\bar{1}$ s is bound to the IgG through the C1q and C $\bar{1}$ r subcomponents, but it can also form a similar bond with another α' chain.

Fig. 5. Comparison of the ability of antibody-antigen aggregates with C $\bar{1}$ bound, antibody-antigen aggregates and antibody-antigen aggregates plus C $\bar{1}$ s to bind C4. Samples (40 μg) of antibody-antigen aggregates with optimal amounts of C $\bar{1}$ bound (Δ), antibody-antigen aggregates ($\bullet$) and antibody-antigen aggregates plus 130 molecules of C $\bar{1}$ s/1000 molecules of IgG ($\circ$) were incubated with increasing concentrations of ^{125}I -labelled C4 in 100 mM-NaCl/2 mM-CaCl $_2$ /10 mM-Tris/HCl, pH 7.4, at 30°C for 30 min. After incubation the aggregates were centrifuged at 2000g for 15 min, washed three times with the NaCl/CaCl $_2$ /Tris/HCl, pH 7.4, buffer and transferred to fresh tubes. Samples were then taken for counting to determine the bound C4.

Nature of C4b attachment to antibody-antigen aggregates

When aggregates with C $\bar{1}$ and C4b are washed with 100 mM-NaCl/5 mM-EDTA/10 mM-Tris/HCl, pH 7.4, all the bound C $\bar{1}$ r and C $\bar{1}$ s, but only

Table 2. Effect on bound C4 of washing antibody-antigen aggregates with buffers designed to remove the C1 subcomponents

Samples of antibody-antigen aggregates with increasing amounts of ^{125}I -labelled C4 bound were washed twice with 100 mM-NaCl/5 mM-EDTA/10 mM-Tris/HCl, pH 7.4, to remove the C1r and C1s subcomponents. The number of ^{125}I -labelled C4 molecules removed was determined by counting. The aggregates were then washed twice with either 1 M-NaCl/20 mM-EDTA/30 mM-acetate, pH 4.5, or 0.7 M-NaCl/20 mM-EDTA/50 mM-Tris/HCl, pH 10, and the amount of C4 removed was determined from the radioactivity in the washes.

C4 bound (molecules/ 1000 molecules of IgG)	Molecules of C4 removed by		
	EDTA Washes	pH 4.5 Washes	pH 10 Washes
0	0	0	0
15	3	1	1.5
65	13	3	7
180	27	11	17

15–20% of the bound C4b, is removed (Table 2). Electrophoresis on SDS/polyacrylamide gels shows that this loss of C4b corresponds with the loss of all the α' chain monomer and some α' chain dimer, but with no loss of the α' -H band. Thus, although there may be some weak residual affinity of either C4b or the C4b dimer for C1r and C1s, the bulk of the C4b on the aggregates is not bound to these subcomponents. Addition of C1r and C1s to the aggregates restores the ability of the aggregates to destroy C3 in the presence of excess C2.

Similarly, if the aggregates were washed subsequently with buffers that removed C1q (1 M-NaCl/0.02 M-EDTA/0.03 M-acetate, pH 4.5, or 0.7 M-NaCl/0.02 M-EDTA/0.05 M-Tris/HCl, pH 10) (Gigli *et al.*, 1976; Arlaud *et al.*, 1979) approx. 7–9% and 12–15% of the bound C4b was removed, respectively. This suggests that the C4b on the aggregates cannot be bound through C1q. Some of the removal of C4b by the buffers containing high salt at high or low pH is probably due to dissolution of the aggregates which, using ^{125}I -labelled IgG, can also be detected in the washes. It is concluded therefore that the most stable binding of C4b to antibody aggregates is through the antibody or antigen. However, as the amount of ovalbumin as seen on the gels is unchanged at all levels of C4 binding, it is improbable that C4 interacts with the antigen.

Site of interaction of C4b with antibody-antigen aggregates

The results of the preceding sections (shown in Figs. 2–4) have suggested that effective C4 molecules on the aggregates may be those which interact with the antibody molecule. This was investigated to determine if the interaction of the α' chain of C4b was with the heavy or light chains of IgG.

Samples of aggregates containing ^{125}I -labelled antibody and with optimal amounts of bound C1 were incubated with 21000 molecules of C4/1000

molecules of IgG at 30°C for 30 min. After washing, duplicate aliquots were electrophoresed without reduction on 6.5% (w/v) polyacrylamide gels in SDS-containing buffers; one duplicate was stained for protein and the other was cut into 2 mm strips for counting. Slices containing the C4b-IgG complex (Fig. 6) were broken into small fragments and eluted with 0.5 ml of 20 mM-dithiothreitol at room temperature for 20 h. The extract and two washes with 0.25 ml of the same solution were combined and the radioactivity was counted, showing a recovery of 70–80% in the eluates. These extracts were freeze-dried and electrophoresed in 9% (w/v) polyacrylamide gels in SDS-containing buffers (Fig. 6) and the radioactivity in the heavy and light chains of IgG was measured.

If no C4 had been added the heavy/light chain ratio of radioactivity was 1.9:1. However, when the eluted C4b-IgG complex was run under reducing conditions the heavy/light chain ratio fell to 1.1:1 and radioactivity was now present in the higher molecular weight band. The radioactivity of the higher molecular weight band, together with the heavy chain, was 1.8 times that of the light chain, which is close to the heavy/light chain ratio in the absence of C4. This result suggests that the C4b in the C4b-IgG complex has bound through its α' chain to the heavy chain of the antibody molecule.

Earlier evidence (Goers & Porter, 1978) suggested that C4 bound to the Fab section of the antibody molecule in the aggregates. The results of the experiment described in that paper showed that the C4 binding capacity of antibody-antigen aggregates was the same as that of aggregates made of 80% (Fab')₂ and 20% IgG (molar ratio) and antigen. This was repeated using ^{125}I -labelled C4. Samples of antibody-antigen aggregates, mixed antibody/(Fab')₂-antigen aggregates and (Fab')₂-antigen aggregates were incubated with 6000 units of C1, and subsequently washed. The whole antibody and mixed antibody/(Fab')₂ aggregates which

Fig. 6. Radioautograph of C4b-¹²⁵I-labelled IgG aggregate

Samples of ¹²⁵I-labelled antibody-antigen aggregates with C4b bound were run in duplicate under non-reducing conditions on an SDS/6.5% (w/v) polyacrylamide-slab gel (Laemmli, 1970). One duplicate was stained for protein, dried down and radioautographed; a line drawing of the radioautograph is shown. The other duplicate was cut into 2mm strips and counted. Slices containing the C4b-IgG complex, and IgG, were broken into small fragments and eluted with 0.5 ml of 20mM-dithiothreitol at room temperature for 20h. The extract and two washes with 0.25 ml of the same solution were combined, freeze-dried, and then electrophoresed under reducing conditions on an SDS/9% (w/v) polyacrylamide slab gel (Laemmli, 1970). The gel was stained for protein, dried down and radioautographed; a line drawing of the radioautograph is shown. The bands containing ¹²⁵I-labelled material were cut out and counted. The radioactivity measured in each band is shown and the ratio of the counts in each band was calculated taking the light chain as 1.00.

were in excess of the C₁ present, were shown by SDS/polyacrylamide gel electrophoresis under non-reducing conditions to have bound approx. the same amounts of C₁q and C₁r. The C₁s bound in both cases could not be compared, as the mobility of unreduced (Fab')₂ was the same as that of unreduced C₁s. (Fab')₂-antigen aggregates appeared to have bound very little C₁ as judged by SDS/polyacrylamide gel electrophoresis. The aggregates were then incubated with increasing concentrations of C4 at 30°C for 30min. After washing, samples were counted, and the results showed that mixed aggregates bound the same amount of C4 as whole aggregates, with aggregates formed from (Fab')₂-antigen alone binding only 15% of that bound by whole aggregates (Fig. 7). This confirms the results of Goers & Porter (1978) and shows that C4 binding to the aggregates correlates with the Fab section present and not the Fc section. The low

binding of C4 to (Fab')₂ aggregates may be due to some C₁ binding, due to a small contamination (<5%) of the (Fab')₂ with whole antibody. This would permit some activation of C4.

When the different aggregates with C4b bound were electrophoresed in SDS/polyacrylamide gels under reducing conditions, the intensity of the α'-H band was decreased and a new band running ahead of the α'-α' band appeared (Fig. 8). It is probably equivalent to an α'-Fd band. This was confirmed using ¹²⁵I-labelled (Fab')₂ in the mixed aggregates and unlabelled C4 when this band was shown to contain (Fab')₂ radioactivity. As expected, very little C4 was seen on (Fab')₂-antigen aggregates.

Molecular weight estimations of unreduced and reduced C4b-IgG and C4b-C4b complexes

The apparent molecular weights of the reduced complexes were estimated by comparison of their

Fig. 7. Binding of C4 to antibody-antigen aggregates, mixed antibody/ $(\text{Fab}')_2$ -antigen aggregates and $(\text{Fab}')_2$ -antigen aggregates

Samples containing $50\mu\text{g}$ of antibody-antigen aggregates (O), $42\mu\text{g}$ of mixed antibody/ $(\text{Fab}')_2$ -antigen aggregates (●) and $40\mu\text{g}$ of $(\text{Fab}')_2$ -antigen aggregates (Δ), were incubated with 6000 units of C1 as described in the Materials and Methods section. The samples were then incubated with increasing concentrations of ^{125}I -labelled C4 in 100mM -NaCl/ 2mM - CaCl_2 / 10mM -Tris/HCl, pH 7.4, at 30°C for 30 min. After centrifugation at $2000g$ for 15 min, the aggregates were washed three times with the NaCl/ CaCl_2 /Tris/HCl, pH 7.4, buffer and transferred to fresh tubes. Samples were then taken for counting to determine the bound C4.

Fig. 8. Electrophoresis in SDS/5% (w/v) polyacrylamide-disc gels of (a) antibody-antigen aggregates and (b) mixed antibody/ $(\text{Fab}')_2$ -antigen aggregates with C1 bound and increasing amounts of C4 added

Gels were run under reducing conditions by the method of Weber *et al.* (1972) on polyacrylamide disc gels. (1) No C4 added; (2) 13 000 molecules of C4 added/1000 molecules of antibody- $(\text{Fab}')_2$; (3) 41 000 molecules of C4 added per 1000 molecules of antibody- $(\text{Fab}')_2$.

rate of movement, relative to that of ovalbumin polymers cross-linked with dimethyl suberimide (Carpenter & Harrington, 1972), on electrophoresis in 4% (w/v) polyacrylamide-disc-gels in SDS-containing buffers (Weber *et al.*, 1972). The α and β chains of C3, α , α' and β chains of C4, phosphorylase, bovine serum albumin and the β 1H protein all fell on the line given by plotting rates of movement and molecular weights for the ovalbumin polymers (Fig. 9), agreeing with earlier reports that

cross-linked ovalbumin is a reliable molecular weight marker (Carpenter & Harrington, 1972; Lumsden & Coggins, 1977). Estimates based on this plot give apparent molecular weights of 160 000 for the α' -H band, 130 000 for the α' - α' band and 120 000 for the α' -Fd band though if single chains were associated the α' -H would give $85\,000 + 50\,000 = 135\,000$, α' - α' 170 000 and α' -Fd $85\,000 + 25\,000 = 110\,000$.

Estimates for unreduced C4b-IgG, C4b-C4b and

C4b-(Fab')₂ are 280 000, 340 000 and 240 000 against expected values of 350 000, 400 000 and 300 000, but, as no markers greater than the ovalbumin hexamer at 267 000 were available, these

Fig. 9. Plot of logarithm of molecular weight against R_F for cross-linked ovalbumin and various standard proteins on electrophoresis in SDS/4% (w/v) polyacrylamide gels. Gels were run by the method of Weber *et al.* (1972). Ovalbumin (O) was cross-linked with dimethyl suberimidate as described by Carpenter & Harrington (1972). Other standard proteins were as described in the Materials and Methods section. The vertical bars indicate the R_F values of (1) the α' -H, (2) the α' - α' and (3) the α' -Fd complexes.

were obtained by extrapolation of the plot and would be expected to be less reliable. However, they do suggest that interactions are between single molecules of C4b and single molecules of antibody or the (Fab')₂ fragments of antibody. No protein other than C4 could be present in the C4b dimer molecule as the same product is formed when C4 is activated in solution with catalytic amounts of C1s.

Nature of the interaction between C4 and antibody-antigen aggregates

As the results of the previous sections have suggested that the α' -H and α' - α' bonds may be covalent in nature, several possibilities were considered.

One was that traces of transglutaminase (factor XIII) might be present in one or other of the serum protein preparations and was catalysing formation of a ϵ -(γ -glutamyl)lysine bond similar to that in a fibrin clot. To test for this, samples of antibody-antigen aggregates with optimal C1 bound were incubated with ¹²⁵I-labelled C4 in the presence of putrescine (0–10 mM), an inhibitor of ϵ -(γ -glutamyl)lysine cross-link formation in fibrin (Folk & Finlayson, 1977). After centrifugation and thorough washing, samples of the aggregates were taken for counting to determine the bound C4b. Pre-incubation of the aggregates with putrescine did not affect the ability of the aggregates to activate and bind C4 (Fig. 10). However, when C4 was incubated with aggregates in the presence of increasing concentrations of putrescine, inhibition of C4 binding occurred (Fig. 10). Half-maximal inhibition was reached at 3.5 mM-putrescine. Similarly, if C4 and C1s were incubated in solution in the presence of

Fig. 10. The effect of increasing concentrations of (a) putrescine and (b) salicylhydroxamic acid on the binding of C4 to antibody-antigen aggregates

Samples (30 μ g) of antibody-antigen aggregates carrying optimal amounts of C1 were incubated with 320 μ g of ¹²⁵I-labelled C4 in 100 mM-NaCl/2 mM-CaCl₂/10 mM-Tris/HCl, pH 7.4, at 30°C for 30 min in the presence of either putrescine or salicylhydroxamic acid (O). After incubation, the aggregates were centrifuged at 2000g for 15 min, washed three times with the NaCl/CaCl₂/Tris/HCl, pH 7.4, buffer, transferred to fresh tubes and taken for counting. Control samples (●) were preincubated with putrescine or salicylhydroxamic acid, washed thoroughly, and then offered ¹²⁵I-labelled C4.

Fig. 11. Effect of putrescine on the dimerization of C4 during cleavage by C $\bar{I}$ s

Samples of (a) ^{125}I -labelled C4 incubated at 37°C for 30 min in 0.1 M-NaCl/2 mM-CaCl $_2$ /10 mM-Tris/HCl, pH 7.4; (b) ^{125}I -labelled C4 incubated as above with 1:40 (w/w) C $\bar{I}$ s. (c) ^{125}I -labelled C4 incubated as above with 1:40 (w/w) C $\bar{I}$ s in the presence of 10 mM-putrescine; were electrophoresed under non-reducing conditions in SDS/3.3% (w/v) polyacrylamide disc gels (Weber *et al.*, 1972). After electrophoresis the gels were sliced into 2 mm segments and counted. The arrows indicate the relative positions of the cross-linked ovalbumin species: (a) monomer; (2) dimer; (3) trimer; (4) tetramer; (5) hexamer.

10 mM-putrescine, inhibition of C4b dimerization occurred (Fig. 11). Cadaverine, another inhibitor of ϵ -(γ -glutamyl)lysine cross-link formation (Folk & Finlayson, 1977) also inhibits C4b dimerization and binding, but is slightly less effective than putrescine (4.5 mM being required for 50% inhibition of C4 binding to aggregates).

Table 3. Incorporation of [^{14}C]putrescine into C4 C4 (0.98 mg) was incubated at 37°C for 30 min in 100 mM-NaCl/10 mM-[^{14}C]putrescine (2.45 Ci/mol)/2 mM-CaCl $_2$ /10 mM-Tris, pH 7.4, with 25 μg of C $\bar{I}$ s. The reaction was stopped by addition of iPr $_2$ P-F to 5 mM, and the mixture was dialysed against 5 $\times$ 500 ml changes of 400 mM-NaCl/20 mM-putrescine/5 mM-EDTA/50 mM-Tris/HCl, pH 7.4. Samples were then taken for scintillation counting. To demonstrate that the putrescine incorporation was covalent, the [^{14}C]putrescine-labelled sample was reduced and alkylated (Gigli *et al.*, 1977), and dialysed against 3 $\times$ 500 ml changes of 0.1% SDS/10 mM-sodium phosphate buffer, pH 6.4. Controls of C4 and C4b were treated in an identical manner except for the addition of C $\bar{I}$ s.

Sample	[^{14}C]Putrescine incorporated (mol/mol of C4)	
	Initially	After reduction and alkylation
C4	0.07	0.04
C4 + C $\bar{I}$ s	0.57	0.49
C4b	0.16	0.08

However, although putrescine can inhibit C4 binding to aggregates it is not due to inhibition of a transglutaminase-type enzyme. This was excluded, as the enzyme from plasma is inactivated by iodoacetamide (Curtis *et al.*, 1973; Chung *et al.*, 1974) and concentrations of iodoacetamide up to 20 mM had no effect on the binding of C4 to aggregates. As transglutaminase could not be catalysing the reaction this suggested that when C $\bar{I}$ s catalysed the activation of C4 a reactive group formed that could form a strong bond with either the heavy chain of IgG or an α' chain of a second C4b molecule. Putrescine, presumably through the amino groups, was a competitive reactant. This was confirmed by incubation of C4 with C $\bar{I}$ s in the presence of 10 mM-[^{14}C]putrescine at 37°C for 30 min. The reaction was stopped by addition of iPr $_2$ P-F to 5 mM to inactivate C $\bar{I}$ s and the mixture was dialysed against five changes of a buffer containing 20 mM-non-radioactive putrescine (0.4 M-NaCl/20 mM-putrescine/5 mM-EDTA/50 mM-Tris/HCl, pH 7.4). The radioactivity of the sample showed that 1 mol of C4 incubated with C $\bar{I}$ s in the presence of [^{14}C]putrescine had bound 0.57 mol of putrescine, while controls of C4 and C4b without C $\bar{I}$ s present had bound 0.07 mol and 0.16 mol of putrescine/mol respectively (Table 3). To demonstrate further that the putrescine incorporation was covalent, [^{14}C]putrescine-labelled C4b was reduced and alkylated (Gigli *et al.*, 1977) and dialysed against 10 mM-sodium phosphate buffer, pH 6.4, containing 0.1% SDS. The radioactivity of the sample showed that greater than 85% of the [^{14}C]putrescine label had remained bound (Table 3).

Salicylhydroxamic acid has been shown to inhibit the reaction of C3, activated by trypsin, with polysaccharides such as Sepharose (T. N. Twose & D. S. Paterson, personal communication). This reagent also inhibited the C4 binding to aggregates (Fig. 10) (0.75 mM being required for 50% inhibition) and C4b dimerization in the presence of C1s, suggesting that the reaction that occurs when C4 is activated may be similar to that which occurs when C3 is activated by trypsin.

Law & Levine (1977), and Law *et al.*, (1979) have shown that activated C3 binds (probably covalently) to erythrocyte surfaces and have given evidence from investigation of its stability that it behaves in a way similar to an ester bond. The stability of the α' -H and α' - α' complexes was investigated by incubating the C4b bound to aggregates at 37°C for 90 min in a solution (0.1 M-NaCl/0.05 M-diethanolamine, pH 11.5) containing either 1% SDS or 1% sodium deoxycholate. Electrophoresis on polyacrylamide gels showed 20–30% release of the α' chain, apparently derived from both the α' -H and α' - α' bands as both had decreased in intensity. Similarly, incubation of the C4b bound to aggregates in 1 M-hydroxylamine at pH 10.5 and 37°C for 1 h caused about 30% release of the α' chain. Incubation of the C3b–polysaccharide complex caused 90% release of the C3b from the polysaccharide under both these conditions in only 30 min (R. B. Sim, personal communication) showing that the C4–IgG bond was significantly more stable than the C3–polysaccharide bond.

Discussion

In a previous paper (Goers & Porter, 1978) it was shown that C4 would bind to antibody–antigen aggregates and to antibody-coated erythrocytes to which C1 had been adsorbed, the maximum amount being some 10–13 times higher in the latter case. However, development of C3 convertase activity in the presence of excess C2, as measured by the capacity to destroy the haemolytic activity of C3, was similar. It was suggested that the convertase enzyme C4₂, formed when C4 and C2 are activated by C1s, bound to the antibody molecules in the aggregates or on the cell surface. Attempts to localize the site of antibody–C4 interaction by splitting the aggregates without destruction of the bound C4, as reported by Chan & Cebra (1968), were unsuccessful. However, when aggregates made from antibody and antigen were compared with those prepared from 80% (Fab')₂/20% IgG antibody and antigen, C4 binding was found to be quantitatively very similar. This was taken as evidence that C4 was interacting with the Fab

regions of the antibody, as removal of 80% of the Fc portion in the aggregates was without effect. Further evidence in support of this view was given by Dodds & Porter (1979) who measured the relative consumption of C1, C2, C3 and C4 on addition of antibody–antigen aggregates, heat-aggregated IgG and heat-aggregated Fc to guinea-pig serum. Heat-aggregated material was less effective than the antibody–antigen aggregates and, with heat-aggregated Fc, equivalent C1 and C4 consumption required 60–80 times greater weight than with antibody–antigen aggregates. Heat-aggregated Fc, however, showed no significant fixation of C2 and C3 (> 1000 less), in agreement with the suggestion that fixation of C4 to Fab is essential for activation of C3 (and of C2). The present work was initiated in order to obtain direct evidence of C4–Fab interaction by using cross-linking reagents. However, it became apparent that ¹²⁵I-labelled C4 remained bound to antibody under denaturing conditions even when no cross-linking reagents were present. When optimum ratios of C1 to antibody were established (Table 1) the ¹²⁵I-labelled C4 bound to aggregates, after extensive washing and subsequent reduction, showed that the β and γ chains were dissociated and present in the expected ratio, but the α' chain was missing (Figs. 1 and 3). Instead, radioactive bands of higher molecular weight were apparent. Repetition using ¹²⁵I-labelled antibody showed that one major high-molecular-weight band (α' -H) also contained IgG, whereas another (α' - α') did not and most of the highest molecular weight material did not. The ratio of radioactivity of the heavy and light chains of IgG was 1.9:1. If the band containing the C4–IgG complex was reduced and re-run on acrylamide gels in SDS-containing buffers, the heavy chain/light chain ratio was now only 1.1:1. The radioactivity in the α' -H band accounted for the missing radioactivity in the heavy-chain band (Fig. 6), i.e. the C4b α' chain is bound to the IgG heavy chain.

When the C4 is bound predominantly in the Fab' portion in the mixed IgG/(Fab')₂ antibody aggregates, a lower molecular weight band appeared, again containing both C4 and Fab' as judged by radioactivity, and hence most likely to be a complex of Fd, the N-terminal half of the heavy chain and the α' chain of C4b. The bond is likely to be between the α' chain of C4 and the first constant domain of the heavy chain, but there is no evidence to confirm this.

The apparent molecular weight of the C4b–IgG complex suggests that this component contains one molecule each of C4b and IgG, and that the main complex containing only C4b contains two molecules of C4b. However, the apparent molecular weights of the peptide chains suggest that the α' - α' band is of lower molecular weight than the α' -H band, though the expected weights are 170000 and

135000 respectively. It can only be suggested that one linkage is formed between α' and H chains and two between α' and α' chains, as the reactive group is in the α' chain, leading to a more compact structure in denaturing conditions and hence higher mobility for the α' dimer.

The finding that the C4-IgG complex forms only when C1s is bound to the IgG in C1, and not when C1s is present in solution with the C4 and antibody aggregates, supports the view that the reactive group formed when C4 is split to give C4b has a short half-life. Unless the C4 is split in close proximity to the IgG, the reactive group formed reacts either with another α' chain or with water. If the antibody was on a cell surface then presumably the activated C4b could also react with the cell surface.

The stability of the linkage formed suggests that it is covalent, but it cannot be that between the γ -CO₂H of glutamic acid and the ϵ -NH₂ group of lysine, the formation of which is catalysed by transglutaminase, as in a fibrin clot, as its formation is not inhibited by iodoacetamide (Folk & Finlayson, 1977). However, the diamines putrescine and cadaverine inhibit the formation of the α' -H bond, and putrescine binds very strongly and probably covalently to the activated C4. A plausible explanation is that the splitting of C4 to C4b by C1s exposes a reactive acyl group. It was suggested by Law *et al.* (1979) that when C3 is activated by C3 convertase to give C3b an active acyl group forms an ester bond with a polysaccharide in the cell surface or with an activator such as zymosan. The α' -H bond formed when C4b binds to antibody appears to be significantly more stable than the postulated C3b-polysaccharide ester bond at high pH or in hydroxylamine and therefore appears unlikely to be an ester bond. The two phenomena are likely to be similar, but presumably the active acyl group perhaps released from a labilized acyl-thiol or acyl-imidazole ester in C4 is reacting with an amino-acid residue in the Fd section of the IgG, not with a sugar residue.

There have recently been reports of C4 (Law *et al.*, 1980), epidermal growth factor (Linsley *et al.*, 1979) and thrombin (Baker *et al.*, 1979) forming strong, perhaps covalent, bonds with cell surfaces, and also of the identification of a γ -carboxyl group of glutamic acid in α 2 macroglobulin reacting with methylamine (Swenson & Howard, 1979). It has been known for many years that human C3, C4 and α 2 macroglobulin can all be inactivated by ammonia and hydrazine (Gordon *et al.*, 1926; Müller-Eberhard & Biro, 1963; Steinbuch *et al.*, 1968). These may all be related reactions, and if so the formation of active acyl groups on splitting of peptide bonds in proteins may prove to be a rather common phenomenon. As [¹⁴C]putrescine reacts with C4 when the latter is activated with C1s it

should be possible to identify the active group in C4b.

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