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The covalent-binding reaction of complement component C3

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The complement protein C3, when activated by limited proteolysis, forms a short-lived reactive intermediate fragment, 'nascent' C3b, which is known to bind covalently to certain surfaces. The characteristics of the covalent binding reaction have been studied by using Sepharose–trypsin as a combined proteolytic activator and binding surface for C3. Binding of C3 to Sepharose–trypsin is saturable, with a maximum of 25–26 molecules of C3b bound per molecule of trypsin. A minimum life-time of about 60 μs for the reactive intermediate has been calculated from binding of C3 at saturation. Initial binding efficiencies of over 30% can be obtained at physiological pH and ionic strength. The efficiency of C3 binding to Sepharose–trypsin decreases as pH increases and also shows a slight decline at high ionic strength. The covalent binding of C3 to Sepharose–trypsin can be inhibited by a range of oxygen and nitrogen nucleophiles. Activation of C3 in the presence of radioactive forms of four such nucleophiles, phenylhydrazine, methylamine, glycerol and glucosamine results in apparent covalent incorporation of the nucleophile into the C3d fragment of C3. The quantity of radioactive nucleophile bound can be predicted from the observed potency of the nucleophile as an inhibitor of the binding of C3 to Sepharose–trypsin. The radioactive nucleophiles may be considered as 'active-site' labels for C3.

The complement system in blood plasma is an effector system involved in the neutralization and clearance of foreign material and cell debris present in the circulation or extracellular fluid (Lachmann, 1979). The system consists of some 20 proteins, which are activated sequentially in response to stimuli such as cell-wall components of micro-organisms and antibody–antigen complexes (Porter & Reid, 1979). A key step in the complement sequence is the activation of the third component, C3, a glycoprotein of 186000 mol.wt consisting of two disulphide-linked polypeptide chains of 116000 (α-chain) and 70000 (β-chain) mol.wt. (Nilsson & Mapes, 1973). C3 is the most abundant of the complement proteins, being present in plasma at a concentration of about 1.2 g/litre. Proteolytic fragments of C3 produced during complement activation have biological activities essential in antigen clearance (Lachmann, 1979). Specific C3 convertase enzymes cleave C3 at a single peptide bond 77 residues from the *N*-terminus of the α-chain to produce two fragments, C3a (8000 mol.wt.) and C3b (178000 mol.wt.) (Fernandez & Hugli, 1977). C3b in serum or plasma is further degraded by specific control proteins, C3b inactivator and β_1H, and by unidentified serine proteinases to form the fragments C3c (140000 mol.wt.), C3d (25000–32000 mol.wt.) and C3e (10000 mol.wt.) (Nagasawa & Stroud, 1977; Pangburn *et al.*, 1977; Rother, 1971). The peptide-bond cleavage that produces fragments C3a and C3b can be mimicked by trypsin, and prolonged digestion of purified C3 with trypsin also produces fragments similar to the physiological products, C3c, C3d and C3e (Bokisch *et al.*, 1969; Tack *et al.*, 1979; Fontaine & Rivat, 1979). The biological activities of these C3 fragments have been reviewed (Lachmann, 1979; Porter & Reid, 1979).

Immediately after cleavage of C3 into C3a and C3b, the 'nascent' C3b fragment possesses a very short-lived activity that enables it to bind strongly to suitable surfaces, e.g. protein or glycoprotein, carbohydrate and phospholipid (Feinstein & Munn, 1966; Capel *et al.*, 1978; Arnaout *et al.*, 1979). This activity is generally referred to as 'acceptor' activity. The acceptor binding of C3b was initially thought to be entirely hydrophobic in nature (Müller-Eberhard,

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; the nomenclature of complement components is that recommended by the World Health Organisation (1968).

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1975; Capel *et al.*, 1978). However, recent evidence indicates that a significant proportion of the C3b is bound by covalent (ester) bonds, of which the carbonyl group is donated by C3 (Law & Levine, 1977; Law *et al.*, 1979*a*,*b*; Twose *et al.*, 1980). In order to investigate the characteristics of the covalent-binding reaction we have studied simplified systems involving the binding of ^{125}I-labelled C3 to Sepharose–trypsin. We have also investigated the binding properties of low-molecular-weight radioactive ligands to 'nascent' C3b as a means of labelling the site on C3 involved in the covalent binding.

In the following paper (Sim & Sim, 1981) we describe autolytic fragmentation of complement component C3, which occurs only with forms of C3 that can participate in the covalent-binding reaction.

Materials and methods

Materials

Outdated human plasma containing citrated-phosphate/dextran anticoagulant was donated by the National Blood Transfusion Service, John Radcliffe Hospital, Oxford, U.K. Plasma was pooled, made 1 g/litre with polybrene (*NNN'N'*-tetramethyl-1,6-hexanediamine polymer with 1,3-dibromopropane), centrifuged (30 min, 15000 *g*) to remove cell debris, and stored at −20°C. All contact with glass was avoided. Serum was prepared from outdated plasma as described previously (Gigli *et al.*, 1976).

The sources of commercially available materials were as follows: DEAE-Sephacel, CM-Sephadex C50, DEAE-Sephadex A50 and Sepharose from Pharmacia (G.B.), Hounslow, Middx., U.K., Deacidite FF from Permutit Co., London, U.K.; materials for polyacrylamide-gel electrophoresis, SDS and hydrazine sulphate from BDH, Poole, Dorset, U.K.; Trypsin (type XI, diphenylcarbamoyl chloride-treated), soya-bean trypsin inhibitor (type I-S), methylamine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, D-(+)-glucosamine hydrochloride, mannose, *p*-nitrophenol *p'*-guanidinobenzoate and chloramine T from Sigma, Poole, Dorset, U.K.; salicylhydroxamic acid, acetohydroxamic acid, polybrene and CNBr from Aldrich Chemical Co., Gillingham, Dorset, U.K.; phenylhydrazine hydrochloride, from May and Baker, Dagenham, Essex, U.K.; and hydroxylamine hydrochloride from Cambrian Chemicals, Croydon, Surrey, U.K. Radioactive materials, namely Na^{125}I (specific radioactivity 16.1 mCi/μg of iodine), [1(3)-(*n*)-^{3}H]glycerol (sp. radioactivity 2.5 Ci/mmol), D-[6-^{3}H]glucosamine hydrochloride (sp. radioactivity 38 Ci/mmol) and [^{14}C]methylamine hydrochloride (sp. radioactivity 40.4 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.; [U-^{14}C]phenylhydrazine hydrochloride (sp. radioactivity 3.03 mCi/mmol) was from ICN, Irvine, CA, U.S.A. Materials for liquid-scintillation counting were purchased from Koch–Light, Colnbrook, Bucks., U.K. Other reagents were from Fisons, Loughborough, Leics., U.K.

Methods

Sepharose–trypsin. Sepharose 4B was activated at pH 10.2 with 1 g of CNBr/5 ml of packed Sepharose as described by Cuatrecasas (1970). Trypsin was coupled to the activated Sepharose at pH 8.9 with 90% coupling yield such that the final concentration of trypsin was 0.4 mg/ml of packed Sepharose. Excess binding sites were saturated with glycine. Sepharose–trypsin was stored as a 1:1 (v/v) suspension in 100 mM-sodium acetate/5 mM-$CaCl_2$, pH 4.5, containing 0.02% (w/v) NaN_3.

The activity of the Sepharose–trypsin, relative to soluble trypsin, in cleaving C3 was compared by determining initial rates of destruction of C3 haemolytic activity by various concentrations of fixed and soluble trypsin. The concentration of the soluble trypsin was determined by titration with *p*-nitrophenol *p'*-guanidinobenzoate (Chase & Shaw, 1970).

Sepharose–IgG. Human IgG, prepared as a side fraction of a C2 and Factor B preparation (Kerr, 1979), was coupled to CNBr-activated Sepharose as described above. The final preparation contained 2 mg of IgG/ml of packed Sepharose.

Preparation of C3. C3 was isolated from outdated human plasma by modifications of the method of Tack & Prahl (1976). Initial steps of purification by 5% (w/v) and 12% (w/v) poly(ethylene glycol) 4000 and passage through Sepharose–lysine were carried out as described by Tack & Prahl (1976). Contact of the plasma with glass was avoided up to and during precipitation. The C3 fraction was then dialysed against 25 mM-potassium phosphate (pH 7.0)/5 mM-EDTA/25 mM-6-aminohexanoate and applied to a column (20 cm × 5 cm diam.) of DEAE-Sephadex A50 in the same buffer. C3 was eluted by a 2-litre linear gradient of NaCl in the same buffer to a limiting concentration of 450 mM-NaCl. C3 was detected in the eluate by assay of haemolytic activity and by analysis of SDS/polyacrylamide gels of column fractions. The C3 pool from DEAE-Sephadex contains as major contaminants C4b-binding protein, β_1H, C5, inter-α-trypsin inhibitor and haemolytically inactive C3, as well as traces of C3b, caeruloplasmin and C4. This material was dialysed against 20 mM-Tris/acetate (pH 6.80)/5 mM-EDTA/10 mM-NaCl/25 mM-6-aminohexanoate and applied to a column (20 cm × 5 cm diam) of CM-Sephadex C50 in the same buffer. C4b-binding protein, inter-α-trypsin inhibitor, caeruloplasmin and inactive C3 were eluted from the column by washing with 1 litre of the starting buffer. Haemolytically active C3 was eluted by applying a

linear gradient (total volume 2 litres) of NaCl in the starting buffer to a final concentration of 300mM-NaCl. C3b, C5 and β_1H are eluted late in the gradient and can be pooled separately from the active C3. If necessary, the C3 pool was concentrated by equilibrating in 25mM-potassium phosphate/5mM-EDTA/25mM-6-aminohexanoate, pH7.0, and binding to a column (20cm × 1.5cm diam.) of DEAE-Sephacel in the same buffer. The C3 was eluted by washing the column with the starting buffer made 250mM with respect to NaCl.

Storage of C3. C3 preparations were stored after rapid freezing as single droplets in liquid N_2. This was done by pumping a C3 solution, in the buffer in which it is eluted from DEAE-Sephacel, through a peristaltic pump at a rate of 75ml/h, using 1.5mm (internal diam.) tubing, such that discrete drops of approx. 50μl volume were formed and dropped directly into liquid N_2. The frozen droplets were stored at −70°C. No haemolytic activity is lost during freezing provided that potassium phosphate buffers are used. We are indebted to Dr. B. F. Tack for suggesting this method of storage.

Preparation of C3b. C3b was prepared from C3 by trypsin cleavage as described by Bokisch *et al.* (1969).

Haemolytic assays of C3. Haemolytic activity of C3 was determined as described by Tack & Prahl (1976).

SDS/polyacrylamide-gel electrophoresis. Slab polyacrylamide gels in buffers containing SDS were run as described by Laemmli (1970). Protein samples were prepared for electrophoresis, and the gels were stained with Coomassie Blue and destained as described previously (Gigli *et al.*, 1976). Radioautography of dried slab gels was done as described by Campbell *et al.* (1980).

Liquid-scintillation counting. Aqueous samples (50–100μl), containing ^{14}C or ^{3}H, were counted for radioactivity in 10ml of 1,4-dioxan/0.5% (w/v) 2,5-diphenyloxazole/2% (w/v) naphthalene in an LKB Wallac 1210 Ultrobeta counter. Counting efficiencies (determined by addition of internal standards) in this system were 94 ± 3% for ^{14}C and 31 ± 2% for ^{3}H.

Iodination of C3. C3 was radioiodinated by a modification of the chloramine-T method (Byrt & Ada, 1969). To 500μg of C3 in 1ml of 125mM-potassium phosphate (pH7.4)/100mM-KCl/1mM-EDTA was added 1mCi (10μl) of Na^{125}I (carrier-free), 100μl of dimethyl sulphoxide and 50μl of chloramine-T (2mg/ml). The reaction was stopped after 4min at 0°C by addition of 200μl of sodium metabisulphite (10mg/ml). Free and bound iodine were separated by gel filtration on a column (12cm × 0.8cm diam.) of Sephadex G-50 (Coarse grade) in the C3 sample buffer. Five to six atoms of ^{125}I were incorporated per 100 molecules of C3.

Assay of binding of C3b to Sepharose–trypsin. Portions (up to 600μl) of a 1:1 (v/v) suspension of Sepharose–trypsin in pH4.5 storage buffer were placed in 2.5ml polystyrene tubes and washed three times by centrifugation (5min, 1000*g*) with 2ml of the assay buffer, 10mM-triethanolamine/HCl/140mM-NaCl/5mM-EDTA, pH7.5. The Sepharose–trypsin was then resuspended in the assay buffer and ^{125}I-labelled C3 (sp. activity 1500–2000c.p.m./μg) in the same buffer was added. Tubes were incubated in a water bath equipped with a shaker, or on a rotatory stirrer. The reaction was stopped by addition of 200μl of soya-bean trypsin inhibitor (10mg/ml in the same buffer). The tubes were centrifuged and a portion of the supernatant was retained for assay of the remaining C3 activity. The Sepharose–trypsin was then washed by centrifugation at 20°C as follows: three washes with 2ml of 2.5mM-sodium barbitone/HCl/72mM-NaCl/0.1% (w/v) gelatin/2.5% (w/v) glucose, pH7.4; 3 × 2ml washes with 10mM-triethanolamine/HCl/2M-NaCl, pH7.4, and 2 × 2ml washes with 0.1M-Tris/HCl/4M-urea/1% (w/v) SDS, pH8.0. The pellet was resuspended in the assay buffer to 400μl and transferred to a fresh tube for estimation of radioactivity. ^{125}I was counted in an LKB Wallac 1270 Rackgamma counter.

Release of C3 bound to Sepharose–trypsin. Sepharose–trypsin, containing bound ^{125}I-labelled C3 (4000–8000c.p.m./100μl packed volume), was prepared and washed to remove non-covalently bound C3 as described above. The Sepharose–trypsin–C3 was then washed with 50mM-sodium phosphate, pH7.0, and dialysed against this buffer with Deacidite FF anion-exchange resin in a separate dialysis sac in order to remove SDS (Lenard, 1971). Portions of 100μl (packed volume) of the Sepharose–trypsin–C3 were then incubated at 37°C in a total volume of 1ml. To investigate release of bound radioactivity, incubation mixtures were centrifuged (5min, 1000*g*), 0.5ml of the supernatant was removed and radioactivity in the supernatant and (supernatant + pellet) fractions was determined.

Interactions of phenylhydrazine, glycerol, glucosamine and methylamine with 'nascent' C3b. Radioactively labelled phenylhydrazine, glycerol, glucosamine and methylamine, diluted with the corresponding unlabelled material were added to solutions of C3 in 10mM-Tris/HCl/150mM-NaCl/2mM-EDTA, pH7.5, to the following final concentrations and specific activities: phenylhydrazine, 1.0mM, 261μCi/mmol; glycerol, 100m,M, 164μCi/mmol; glucosamine, 35mM, 71μCi/mmol and methylamine, 50mM, 10μCi/mmol. The C3 solutions were pre-equilibrated to 37°C and digested with 4% (w/w) trypsin at 37°C for 4h. This treatment generates 'nascent' C3b, then degrades C3b to the C3c and

C3d fragments (Bokisch *et al.*, 1969). The reaction was stopped by addition of a 4-fold molar excess over trypsin of soya-bean trypsin inhibitor. The digests were then dialysed against 50mM-NH_4HCO_3, pH 7.9, and freeze-dried. Freeze-dried material was redissolved in 10ml of 100mM-NH_4HCO_3 and the C3c and C3d fragments separated on a column (95cm × 2.5cm diam.) of Ultrogel AcA 34 in 100mM-NH_4HCO_3, pH 7.9. Fractions containing C3c and those containing C3d were pooled separately and freeze-dried. Samples of the C3c and C3d fractions from each incubation were then dissolved in 5–10ml of 8M-guanidine hydrochloride, pH 7.0, with 40mM-dithiothreitol, and reduced by incubation at 37°C for 1h. Sodium iodoacetate was added to a final concentration of 100mM and incubation was continued for 20min at 37°C. The samples were then subjected to gel filtration on a column (95cm × 1.5 cm diam.) of Sephadex G-150 in 8M-guanidine hydrochloride, pH 7.0. Fractions containing C3d or polypeptide chains of C3c were pooled, dialysed against 50mM-NH_4HCO_3, pH 7.9, to remove guanidine, and then freeze-dried. Radioactivity associated with the final pooled material was determined after redissolving the material in 100mM-NH_4HCO_3, pH 7.9. Control experiments were performed in which pre-formed C3b was digested with trypsin in the presence of radioactive materials. Protein from control experiments was subjected to chromatographic procedures as described above.

Results

C3 preparation

C3 was initially isolated by the method of Tack & Prahl (1976), and high yields of good material were obtained in agreement with the original authors. The use of outdated human plasma, however, rather than fresh plasma, was found to result in slight heterogeneity of the C3. Final preparations contained a variable proportion of haemolytically inactive C3 (von Zabern *et al.*, 1980), which although indistinguishable from active C3 on SDS/polyacrylamide gels, could not be cleaved in solution by C3 convertase enzymes. A small percentage of C3 cleavage products, likely to result from the action of C3b inactivator and β_1H on inactive C3 (Harrison & Lachmann, 1980; Crossley & Porter, 1980) was also present. The modifications of the original procedure that we describe reduce this heterogeneity and permit isolation of fully active intact C3. The yield from this procedure is in the range 120–150mg of C3/litre of anticoagulant-diluted plasma. Storage of this material at 4°C results in reappearance of traces of inactive C3. Storage at −70°C, as described in the Materials and methods section, is effective in minimizing inactivation, with only 20% inactivation after storage for 1 year.

Sepharose–trypsin

The fixation of trypsin to the porous structure of Sepharose 4B is likely to result in much of the enzyme being fixed in positions inaccessible to large substrates such as C3, and additional losses of trypsin activity may be caused by damage to the molecule during fixation (Zaborsky, 1973). The amount of the fixed trypsin still active in cleaving C3 was estimated as described in the Materials and methods section. It was found that the Sepharose-bound trypsin was $0.52 \pm 0.05\%$ (mean ± S.D. of four preparations) as active as the equivalent molar quantity of soluble trypsin in cleaving C3. This low percentage activity is predictable from other reports of activities of immobilized-trypsin preparations against protein substrates (Bernfield & Wan, 1963; Axen & Ernback, 1971; Mezzasoma & Turano, 1971). Thus the Sepharose–trypsin preparations used in the present study, although containing 400μg of total trypsin/ml of packed Sepharose, were assumed to contain only 2.1μg of trypsin active in cleaving C3/ml of packed Sepharose.

Characteristics of the C3 binding reaction

Specificity of binding. The adequacy of the washing procedure in removing non-specifically or weakly associated ^{125}I-labelled C3 or C3b from Sepharose is shown in Fig. 1. No ^{125}I radioactivity was associated with Sepharose–IgG, unmodified Sepharose 4B or Sepharose–trypsin pretreated with soya-bean trypsin inhibitor. Association of C3 with Sepharose–trypsin was clearly demonstrable. This binding increases with time and appears saturable. No binding occurs when preformed C3b is incubated with Sepharose–trypsin. Incubation of C3 with Sepharose 4B plus soluble trypsin does not produce significant binding of C3 under the conditions shown in Fig. 1.

Time course and efficiency of C3 binding to Sepharose–trypsin. The binding curve for C3 to Sepharose–trypsin, shown in Fig. 1, is further interpreted in Fig. 2. The binding of C3 approaches saturation when about 10% of the total C3 supplied is bound. This is not due to limitation of C3, as at this incubation time a large proportion of the C3 is still uncleaved. The saturation is therefore likely to represent a limitation in suitable available binding sites for C3. Since the ability of cleaved C3 ('nascent' C3b) to bind to surfaces is short-lived, it is thought that C3b binds in clusters around the site of fixation of the cleaving enzyme (for discussion, see, e.g., Lachmann, 1973). It is therefore likely that the apparent saturation of binding sites is related to the half-life of the 'nascent' C3b, and represents saturation of the sites in close proximity to the bound trypsin molecules. As seen in Fig. 2, the binding efficiency of C3 decreases as more C3b is fixed,

consistent with the interpretation that C3b molecules fixed later in the time course are fixed further from the cleaving enzyme. The apparent number of trypsin molecules accessible to C3 has been calculated, as described above, and the approach to saturation of binding sites corresponds to fixation of about 20 molecules of C3b per trypsin molecule (Fig. 2).

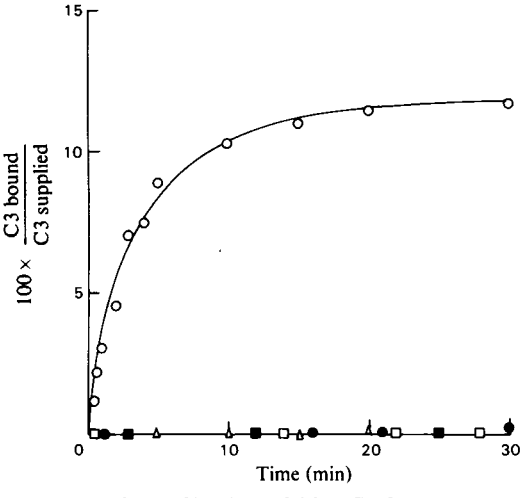

Fig. 1. *Specificity of binding of C3 to Sepharose–trypsin*
^{125}I-labelled C3 (final concn. 850μg/ml) was incubated at 20°C with 250μl (packed volume) of (*a*) Sepharose–trypsin (○), (*b*) unmodified Sepharose 4B in the presence of 2μg of soluble trypsin/ml (●), (*c*) Sepharose–trypsin preincubated (5min, 37°C) with a 50-fold molar excess of soya-bean trypsin inhibitor (□), or (*d*) Sepharose–IgG (■). C3b at the same concentration (850μg/ml) was incubated with Sepharose–trypsin (△). The total reaction volume in each case was 750μl, and incubation buffer and washes were as described in the Materials and methods section.

Effect of varying C3 and Sepharose–trypsin concentrations. The effect of varying C3 and Sepharose–trypsin concentrations on binding efficiency and on total C3 binding are shown in Figs. 3(*a*) and 3(*b*). For simplicity in interpretation, incubation was continued sufficiently long in the experiments shown to consume all the available C3. Increase in C3 concentration leads to greater binding of C3, but efficiency of binding is decreased. Increasing the amount of Sepharose–trypsin similarly leads to a greater percentage of C3 becoming fixed. Both Figs. 3(*a*) and 3(*b*) indicate that binding approaches a maximum at about 21–25 molecules of C3b bound/molecule of active trypsin.

Data from Figs. 3(*a*) and 3(*b*) have been recalculated (Fig. 4) to demonstrate relationships between the amount of C3 supplied per unit of trypsin, the amount of C3 bound per unit of trypsin and the C3 binding efficiency. It is clear from Fig. 4 that the amount of C3b bound is simply related to the amount supplied. Binding reaches an absolute limit of about 26 molecules of C3b bound per molecule of active trypsin. This amount of binding is achieved only at very high C3 input (about 4000 molecules of C3 per active trypsin molecule). The maximum binding efficiency observed is 30–35% and this occurs at low C3 input (less than 25 C3 molecules per active trypsin molecule). It should be noted that the exact form of the binding curve at very low C3 input is uncertain, due to increased error in determining low amounts of C3 bound. Data from Fig. 2 also fit this binding curve if the

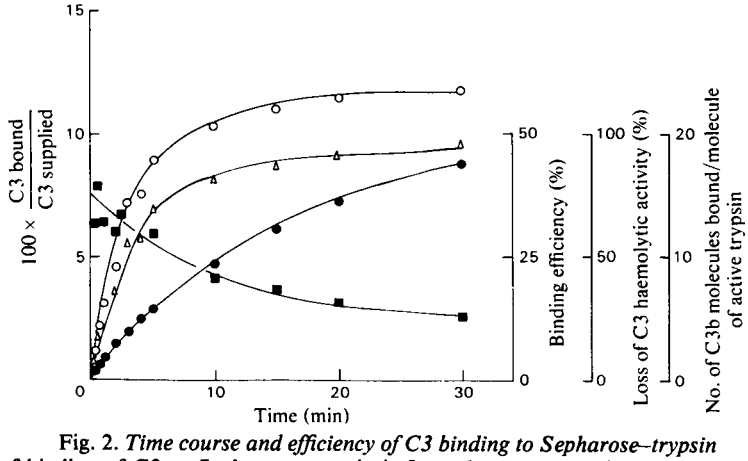

Fig. 2. *Time course and efficiency of C3 binding to Sepharose–trypsin*
The time course of binding of C3 to Sepharose–trypsin is from the same experiment as that shown in Fig. 1. The percentage of the total C3 offered that is bound (○) is shown. The rate of loss of C3 haemolytic activity was also measured (●). The binding efficiency calculated from [total C3 bound (%)]/[C3 cleaved (%)] (■) and the number of C3 molecules bound per molecule of active trypsin (△) have been calculated.

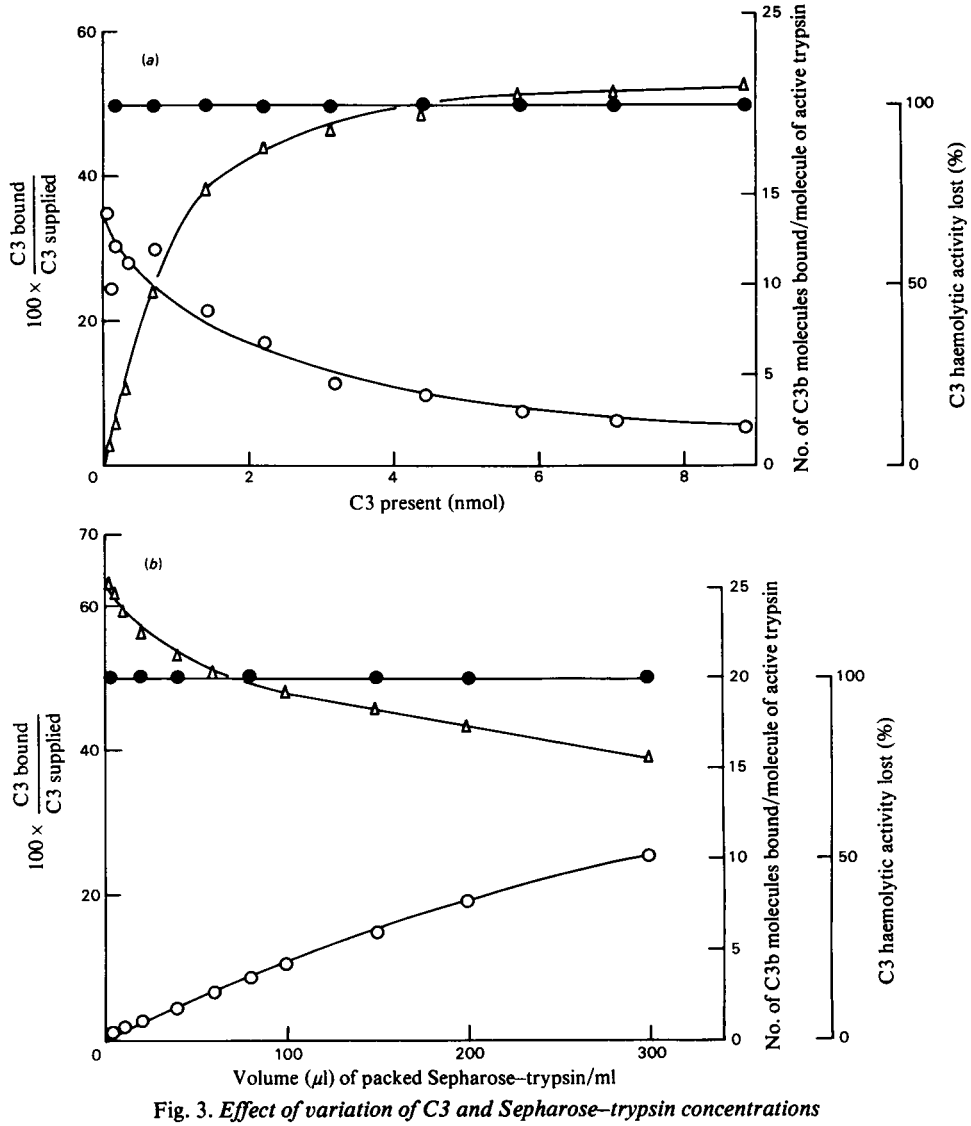

Fig. 3. *Effect of variation of C3 and Sepharose–trypsin concentrations*

(*a*) Various concentrations of ^{125}I-labelled C3 were incubated for 2h at 20°C with 250μl (packed volume) of Sepharose–trypsin. Total incubation volume was 1.25ml. (*b*) Various packed volumes of Sepharose–trypsin were incubated for 4h at 20°C. Total reaction volume was 1.0ml and the final ^{125}I-labelled C3 concentration was 300μg/ml. The percentage C3 bound (C3 bound/C3 supplied × 100) (○) and the loss of C3 haemolytic activity (●) were measured. The number of molecules of C3b bound per active trypsin molecule was calculated (△).

abscissa is plotted as the molar ratio of C3 cleaved (rather than C3 supplied) to trypsin. Thus, in this simple C3-binding system, initial binding efficiency is essentially independent of C3 or Sepharose–trypsin concentration, but overall binding efficiency can be predicted simply from the ratio of C3 molecules cleaved per molecule of trypsin. This is entirely consistent with a model in which 'nascent' C3b has a short half-life, and initial binding occurs with relatively high efficiency close to the cleaving enzyme. As these sites become occupied, and the distance to the closest available sites increases, the probability of binding decreases, and the observed binding efficiency diminishes. The very localized binding of C3b is emphasized by the fact that addition of unmodified Sepharose to the Sepharose–trypsin does not increase the quantity of C3 bound.

Effect of pH on C3 binding. The variation in C3 cleavage, overall binding and binding efficiency with pH is shown in Fig. 5. Overall binding and binding

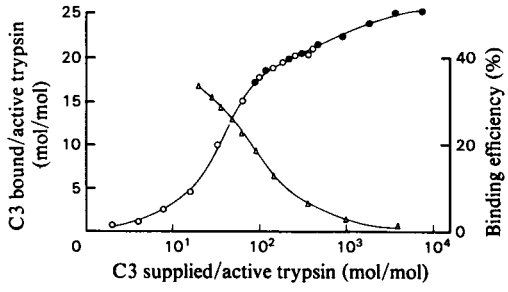

Fig. 4. *Relationship between C3b bound per active trypsin molecule and C3 offered per active trypsin molecule*
Data from Figs. 3(*a*) (○) and 3(*b*) (●) have been expressed as molar ratios of C3 offered per active trypsin molecule and C3b bound per active trypsin molecule. From this, a curve of binding efficiency (i.e. percentage of C3 offered that is bound) has been calculated (△). The abscissa has been expressed on a logarithmic scale to accommodate the wide range of C3/trypsin ratios used.

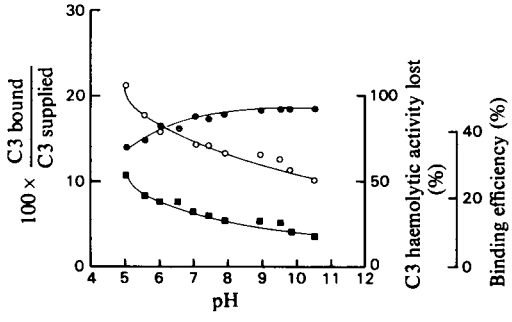

Fig. 5. *Effect of pH on C3 binding to Sepharose–trypsin*
Sepharose–trypsin (125 μl packed volume) was incubated 30 min at 20°C with ^{125}I-labelled C3 (final concn. 120 μg / ml) in 25 mM-Tris / 25 mM-sodium acetate/25 mM-sodium phosphate/75 mM-NaCl, pH 5.0–10.5. Total reaction volume was 2.1 ml. The percentage of C3 offered that binds (○), the percentage of C3 haemolytic activity lost (●) and the binding efficiency (■) (defined in the legend to Fig. 2) were determined.

efficiency are greatest at low pH, and decrease gradually and regularly with increasing pH. The solubility of C3 declines rapidly below pH 5.0 and so values below this range were not investigated. To eliminate the possibility that the unusual pH profile is an artefact of surface charge on the modified Sepharose, the binding of C3 in whole human serum to unmodified Sepharose 4B was investigated in the same pH range. Sepharose acts as an activator of the alternative pathway of complement and so incubation of Sepharose in serum results in C3 deposition on the Sepharose. Experimental conditions were similar to those described by Pepys *et al.* (1976). Results are shown in Fig. 6 and correspond closely to those in Fig. 5. The efficiency of binding of ^{125}I-labelled C3 to EAC$\overline{142}$ cells also shows a similar pH-dependence. (R. S. Freckleton & R. B. Sim, unpublished work). Thus although the rate of C3 cleavage, whether by trypsin or by classical or alternative-pathway convertases varies widely in the pH range studied, binding efficiency increases as pH decreases. This may be an effect of differential decrease by protonation in the nucleophilic properties of the hydroxy groups on the surface to which C3 binds, and of water molecules that compete with surface hydroxy groups to react with C3 (Law *et al.*, 1979*b*; Twose *et al.*, 1980).

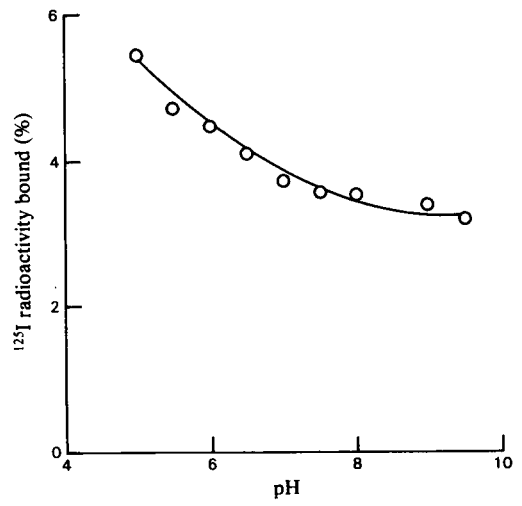

Fig. 6. *Effect of pH on binding of serum C3 to Sepharose 4B*
^{125}I-labelled C3 (10 μl; 10 μg; 930000 c.p.m.) was added to samples (10 ml) of human serum. The serum samples were adjusted to various pH values in the range 5.0–9.5 by addition of 0.15 M-HCl or -NaOH. Each sample was made up to 15 ml volume with 0.15 M-NaCl and mixed with 10 ml of a suspension of Sepharose 4B in 0.15 M-NaCl (1 vol. of packed Sepharose/vol. of NaCl). Samples were incubated 1 h at 37°C, then centrifuged and washed as described in the Materials and methods section.

Effect of ionic strength on C3 binding. The effect of variation in relative salt concentration from 19 to 800 mM on the efficiency of C3 binding is shown in Fig. 7. (A solution with a relative salt concentration of *x* mM has the same conductivity as *x* mM-NaCl.) Binding efficiency shows a gradual decline with increasing ionic strength, but the overall effect is small. Shin & Mayer (1968) have also observed that variation in ionic strength from 0 to 150 mM has negligible effect on C3 binding to EAC$\overline{142}$ cells. Thus charged groups do not appear to play an important role in the binding reaction.

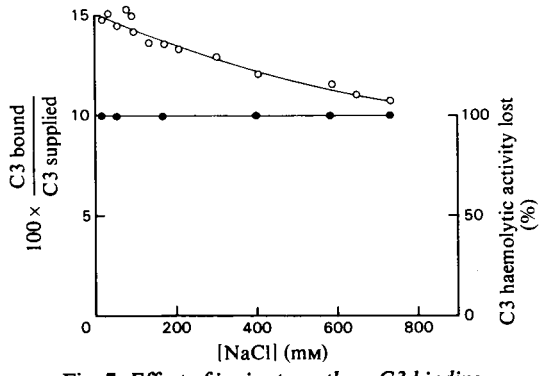

Fig. 7. *Effect of ionic strength on C3 binding*
^{125}I-labelled C3 (final concn. 110μg/ml) was incubated with Sepharose–trypsin (125μl packed volume) for 1h at 20°C in 20mM-Tris/HCl (pH 7.5)/0–750mM-NaCl. Total volume was 2.35ml. The proportion of the C3 supplied that was bound (○) and the loss of C3 haemolytic activity (●) were measured.

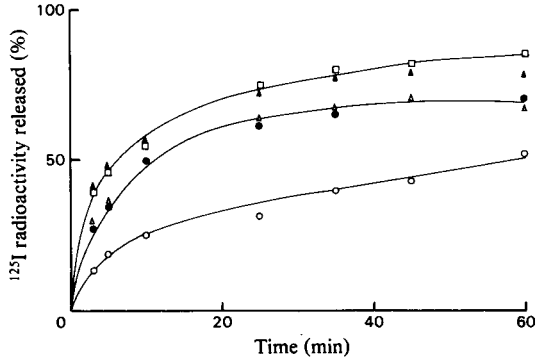

Fig. 8. *Effect of detergents on the rate of release of bound C3b*
Sepharose–trypsin, containing bound ^{125}I-labelled C3b, was incubated at 37°C with 50mM-diethanolamine/HCl (pH 11.5)/100mM-NaCl (○), or with the same buffer containing Lubrol (1%, w/v) (●), Tween 80 (1%, v/v) (△), Nonidet P40 (1%, v/v) (▲), or sodium deoxycholate (1%, w/v) (□). Release of bound ^{125}I was determined at various times as described in the Materials and methods section.

Release of bound C3

In agreement with results presented by Law & Levine (1977) and Law *et al.* (1979*a*,*b*), C3 bound to surfaces may be released by treatment with high concentrations of strong nucleophiles (e.g. 1M-NH_2OH) and by incubation at high pH. Incubation for 1h at pH 7.5 and 37°C with chaotropes such as KBr (1M) or KCNS (1M), which are known to inactivate C3 in solution (Müller-Eberhard *et al.*, 1966), does not release bound C3. Non-ionic and ionic detergents, e.g. Triton X-100 (1%, v/v), Nonidet P40 (1%, v/v) and sodium deoxycholate (1%, w/v), and urea (6M) are without effect at pH 7.5.

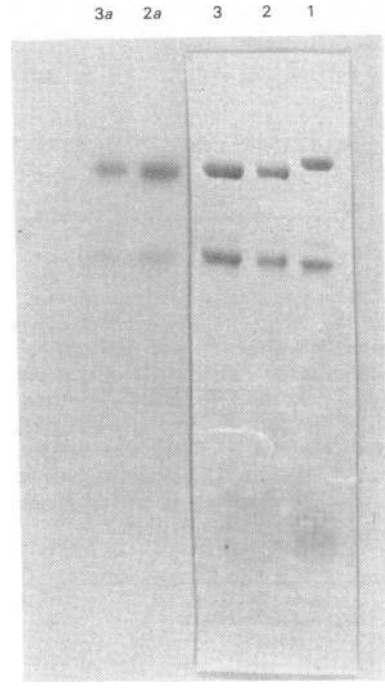

Fig. 9. *Characterization of ^{125}I-labelled C3 released from Sepharose–trypsin*
Sepharose–trypsin containing bound ^{125}I-labelled C3 was incubated for 1h at 37°C in 50mM-diethanolamine/HCl (pH 11.5)/100mM-NaCl, or with the same buffer containing Triton X-100 (1%, v/v). The supernatant from incubation was dialysed against 100mM-NH_4HCO_3 and freeze-dried. The freeze-dried material was reduced and alkylated and run on an SDS/polyacrylamide slab gel; the gel was stained with Coomassie Blue, dried and subjected to radioautography, as described in the Materials and methods section. Tracks 1, 2 and 3 show Coomassie Blue staining and tracks 2*a* and 3*a* are radioautographs. Samples shown are: 1, non-radioactive C3 standard; 2 and 2*a*, material eluted at pH 11.5 without detergent; 3 and 3*a*, material eluted at pH 11.5 with Triton X-100.

The most convenient conditions for release of C3 bound to Sepharose–trypsin were found to be incubation at 37°C with 50mM-diethanolamine/HCl/100mM-NaCl, pH 11.5. The presence of detergents in this buffer greatly enhanced the rate of release (Fig. 8). The detergents Tween 80 and Lubrol caused a 2.5–3-fold enhancement of initial rate of release, whereas Nonidet P40 and sodium deoxycholate caused a 6-fold enhancement of rate

compared with the pH 11.5 buffer alone. Triton X-100 (1%, v/v) and SDS (1%, w/v) gave the same enhancement of release as Nonidet P40 and sodium deoxycholate.

^{125}I radioactivity released from Sepharose–trypsin–C3 by treatment with 50mM-diethanolamine/HCl/100mM-NaCl, pH 11.5, or by the same buffer containing detergent is in the form of C3b (Fig. 9). Thus C3b, once bound, does not undergo significant degradation by bound trypsin. Similar release experiments, in agreement with Law *et al.* (1979*b*) and Arnaout *et al.* (1979) confirm that the C3b is attached to the Sepharose by a bond formed to the C3d portion of the α'-chain of C3b, since treatment of the bound C3b with trypsin (1mg/ml, 30min, 37°C) causes release of C3c, and subsequent incubation of the bound material at pH 11.5 liberates C3d into solution (results not shown).

Inhibition of C3 binding to Sepharose–trypsin

Inhibition of the binding of C3 to Sepharose–trypsin by a wide range of nitrogen, oxygen and sulphur nucleophiles has been examined (Twose *et al.*, 1980), and the implications of this inhibition have been discussed. It appears likely that such inhibitors react directly with the postulated electron-deficient carbonyl group (or acylium ion) present in the C3d region of 'nascent' C3b, forming covalent bonds. The products of reaction of the most potent of such inhibitors (hydroxamic acids, hydroxylamines, hydrazines and thiols) are likely themselves to be insufficiently stable to survive peptide-fractionation procedures and protein-sequencing reactions required for further characterization of the binding site. A number of weakly nucleophilic hydroxyl- and amine-containing compounds were therefore investigated for their capacity to inhibit C3 binding, since it was considered likely that these would form stable amide and ester bonds with 'nascent' C3b. Such compounds would therefore serve as a stable 'active-site' label for C3. Inhibition of C3 binding to Sepharose–trypsin by these compounds is shown in Table 1. Some of the more potent inhibitors are included for comparison. The strong nucleophile inhibitors all cause 50% inhibition of C3 binding at less than 2mM. Several of the weak nucleophiles tested also inhibit binding, but at relatively high concentrations. Putrescine and cadaverine, which inhibit the binding reaction of complement component C4 at concentrations around 4mM (Campbell *et al.*, 1980), are considerably less effective in inhibition of C3 binding. Mannose and glucosamine, in agreement with Capel *et al.* (1978), are also weak inhibitors of the binding reaction, as is glycerol. None of the compounds shown in Table 1 caused significant inactivation of C3 during incubation, although it is known that several, e.g. NH_4Cl, hydrazine and hydroxylamine, inactivate C3 when C3 is treated with higher concentrations of these substances than used for inhibition of binding (Müller-Eberhard & Biro, 1963; von Zabern *et al.*, 1980).

Table 1. *Inhibition of C3 binding to Sepharose–trypsin*

Sepharose–trypsin (300μl packed volume) was incubated (30min, 37°C) with 25μg of ^{125}I-labelled C3 (10800c.p.m.) with a range of concentrations of inhibitors in 10mM-triethanolamine/HCl/150mM-NaCl/5mM-EDTA, pH 7.5. The reaction volume was 1ml. The reaction was stopped and binding of C3 assessed as described in the Materials and methods section. The concentration of inhibitor causing 50% inhibition of C3 binding was determined graphically. C3 cleavage was complete in all cases. Control incubations without Sepharose–trypsin were performed under identical conditions to determine inactivation of C3 by the compounds used.

Effector	Concentration causing 50% inhibition (mM)
Strong nucleophiles	
Salicylhydroxamic acid	0.055
Acetohydroxamic acid	0.8
Hydroxylamine	0.9
Hydrazine	1.8
Phenylhydrazine	0.7
Hydroxyl and amino nucleophiles	
Ammonium chloride	No effect at 100mM
Methylamine	55
Putrescine	52
Cadaverine	145
Glycine	No effect at 100mM
Glucosamine	38
Mannose	100
Glycerol	62

Table 2. *Incorporation of radioactive nucleophiles into C3 fragments*

The incorporation into C3 of radioactive nucleophiles during trypsin digestion was carried out as described in the Materials and methods section. The percentage inhibition of C3 binding to Sepharose–trypsin obtained at the concentration of radioactive species used here (row *b*) was determined from the experiments described in Table 1. Values for incorporation of radioactivity (rows *f* and *g*) were those determined after gel filtration in guanidine hydrochloride. Recovery of radioactivity at each dialysis and gel-filtration step was greater than 95%. 0, not detectable.

	[^{3}H]Glycerol	[^{3}H]Glucosamine	[^{14}C]Methylamine	[^{14}C]Phenylhydrazine
(*a*) Final concentration of inhibitor (mM)	100	35	50	1.0
(*b*) Percentage inhibition of C3b binding to Sepharose–trypsin	60	45	48	90
(*c*) C3 used for digest (nmol)	225	310	850	150
(*d*) Expected d.p.m. incorporated if binding is 1 mol of nucleophile/mol of C3	81500	49100	18870	86200
(*e*) Row (*b*) × row (*d*)	48900	22100	9060	77580
(*f*) Actual d.p.m. incorporated into C3d	55300	26050	8200	81700
(*g*) Actual d.p.m. incorporated into into C3c	0	0	0	0

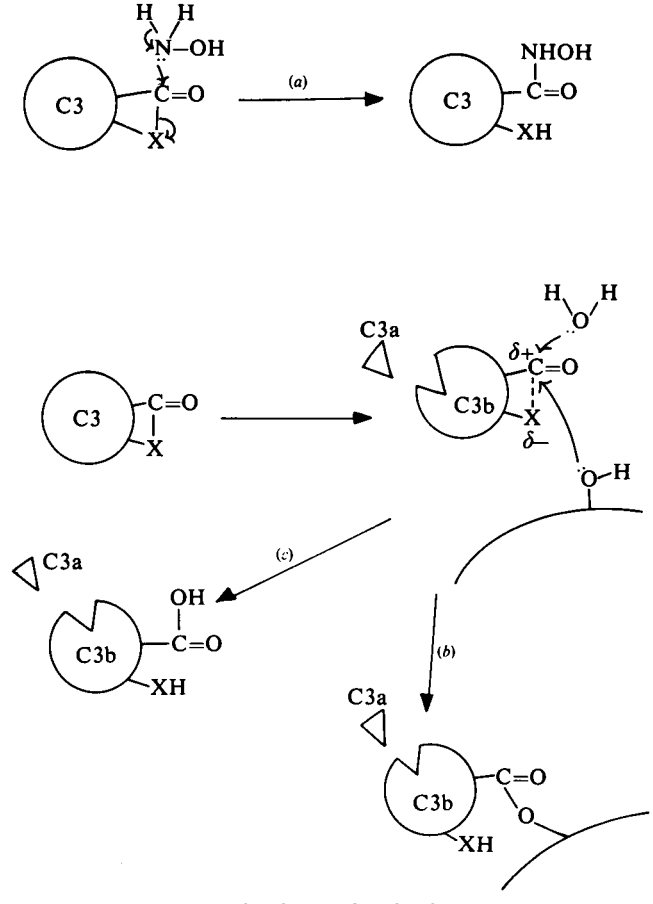

Fig. 10. *A scheme for the covalent-binding reaction of C3*

Details are discussed in the text. (*a*) Chemical inactivation of C3; (*b*) covalent binding of C3b; (*c*) hydrolysis and inactivation of 'nascent' C3b.

Incorporation of inhibitors of binding into 'nascent' C3b

Of the inhibitors shown in Table 1, four were chosen on the basis of their potency and the commercial availability of radiolabelled forms. The incorporation of glycerol, glucosamine, methylamine and phenylhydrazine into 'nascent' C3b was determined as described in the Materials and methods section. Results are shown in Table 2.

Significant binding of radioactivity was observed in the C3d fragment of C3. The C3d fragment, as discussed above, is known to contain the site responsible for the C3 covalent-binding reaction. No significant incorporation was observed in C3c (Table 2, line *g*). In control experiments in which preformed C3b was digested with trypsin as described in the Materials and methods section, there was no significant incorporation of radioactivity into either the C3c or the C3d fragments. This suggests that incorporation of radioactivity occurs only if 'nascent' C3b is generated in the presence of the radioactive nucleophiles.

In the case of phenylhydrazine, the incorporation of radioactivity (Table 2, line *f*) is very close to the amount expected for binding of 1 mol of nucleophile/mol of C3 (Table 2, line *d*). Phenylhydrazine, at the concentration used, would inhibit approx. 90% of the binding of C3 to Sepharose–trypsin. For methylamine, which at the concentration used in the labelling experiment would cause 48% inhibition of C3 binding to Sepharose–trypsin, overall incorporation into the C3d fragment is 0.43 mol of methylamine/mol of C3. Similarly, for glycerol (60% inhibition of binding), incorporation is 0.68 mol/mol of C3 and for glucosamine (45% inhibition of binding) incorporation is 0.53 mol/mol of C3. Thus incorporation of each species matches closely the value predicted from its potency in inhibition of the C3 covalent-binding reaction. This is consistent with the suggestion of Twose *et al.* (1980) that nucleophilic species inhibit the C3 binding reaction by competing with water and surface-bound nucleophiles for 'discharge' of the reactive carbonyl group in 'nascent' C3b. A possible scheme for these reactions is shown in Fig. 10. The binding of radioactive species demonstrated here is resistant to reduction and alkylation of the protein and to denaturation in guanidine hydrochloride and may therefore represent a covalent labelling of an 'active site' in C3b.

High concentrations of certain nucleophiles, e.g. hydroxylamine, react with aspartyl–glycine bonds in proteins (Bornstein & Balian, 1977). High concentrations of nucleophiles are also known to compete with water for discharge of proteolytic-enzyme–substrate complexes in a stereospecific reaction (Fersht *et al.*, 1973). Both of these reactions would result in covalent incorporation of nucleophiles into protein. However, the lack of incorporation of radioactivity in control experiments indicates that these reactions do not contribute significantly to the labelling observed.

Discussion

The use of Sepharose–trypsin as a system for studying C3 binding has several advantages, in that the C3-cleaving enzyme, in this case trypsin, is stable, in contrast with the unstable physiological C3 convertases. Sepharose as a binding surface is relatively homogeneous and is sufficiently stable to survive rigorous washing and elution procedures.

C3 binds efficiently to Sepharose only when cleaved by a proteinase already bound on the surface (Fig. 1). In agreement with Capel *et al.* (1978), cleavage by soluble trypsin in the presence of Sepharose results in very little binding. Provision of additional surface area for binding, without increase in the amount of bound trypsin, does not increase the binding of C3.

Radioautography of ^{125}I-labelled material released from Sepharose at high pH (Fig. 9) confirms that C3 is bound to the Sepharose in the form of C3b and that little further degradation of C3b occurs once it is bound. This finding justifies the calculation of the percentage of C3 bound as a simple percentage of the radioactivity supplied without correction for radioactivity lost by degradation of C3. The C3a fragment, which is separated from bound C3b by washing at high ionic strength, contains less than 5% of the total radioactivity present in C3 under the conditions of radioiodination reported here. No correction was made for loss of C3a.

The study of the general characteristics of the binding reaction (Figs. 2, 3 and 4) demonstrates that binding is saturable, with an apparent maximum of 25–26 covalently bound C3b molecules per molecule of active trypsin. Binding efficiency is initially high (30–35%) but declines as more C3b is fixed. The total amount of C3b bound and the binding efficiency can be readily calculated from the molar ratio C3 cleaved/trypsin. This behaviour is consistent with a model in which the 'nascent' C3b has a limited lifetime, and if it does not bind rapidly to a surface, it loses the capacity to bind. An order-of-magnitude estimate of the lifetime of the reactive grouping in C3b may be made from the binding data presented in Fig. 4. By using values of 4.8 nm (48 Å) for the Stokes' radius of C3b and a diffusion coefficient of 4.5×10^{-7} cm^2/s [calculated from the data of Tack & Prahl (1976) and Gotze & Müller-Eberhard (1970)] and assuming that the packing of C3b around trypsin molecules may be represented by closest packing of spheres, the last of the 25–26 C3b molecules to bind will be at a minimum distance of 28–30 nm (280–300 Å) from

the trypsin molecule. The time taken to diffuse this distance is about 60 μs. Although representation of the bound C3b as a close-packed cluster necessitates a number of simplifying assumptions, the cluster proposed here is very similar in size to that reported by Mardiney *et al.* (1968). These authors conducted an electron-microscopic study of C3b bound to sheep erythrocyte membranes and reported the presence of clusters of C3, of radius 20–40 nm (200–400 Å), containing 10–40 C3 molecules.

A scheme for the activation and binding of C3 is shown in Fig. 10. Present evidence suggests that native C3 possesses a moderately reactive esterified carbonyl group that becomes involved in the covalent binding to surfaces (Law & Levine, 1977; Law *et al.* 1979*b*; Twose *et al.*, 1980). The presence of such a group in native C3 suggests a mechanism for the known inactivation of C3 by amines and strong nucleophiles (Fig. 10, top). It is suggested that cleavage of C3 to C3b causes polarization or possible fission of a bond to this carbonyl group, making the carbonyl group more electrophilic. The reactive carbonyl group may then be attacked by mild nucleophiles on the binding surface, resulting in binding of the C3b, or may be discharged by reaction with water. Since a free thiol group is revealed on conversion of C3 into C3b (Janatova *et al.*, 1979; Tack *et al.*, 1980), X in Fig. 10 may be a sulphur atom. Thus the relevant carbonyl group in C3 may be involved in a thioester, and, as previously suggested, (Law *et al.*, 1979*b*), the binding reaction of C3b may represent a transesterification. The inactivation of native C3 by amines and strong nucleophiles is consistent with the behaviour of a thioester.

Covalent labelling of 'nascent' C3b with methylamine as well as with glycerol (Table 2) implies that C3 can form amide bonds as well as ester bonds. C4, which also possesses an apparent covalent binding activity, has been reported to bind to antibody–antigen aggregates by a bond that is more resistant to alkaline hydrolysis than a simple ester bond (Campbell *et al.*, 1980). This bond may be an amide linkage. It has also been shown that putrescine can be bound covalently to 'nascent' C4b, presumably by an amide bond (Campbell *et al.*, 1980). A study of C4 binding to the erythrocyte surface, however, suggests that C4b forms predominantly ester bonds with membrane constituents (Law *et al.*, 1980). Another protein that has many similarities to C3 and C4, namely α_2-macroglobulin, appears to form covalent bonds with proteinases (Harpel & Hayes, 1979) and to incorporate methylamine covalently by amide linkage to the side-chain carbonyl group of a glutamic acid residue (Swenson & Howard, 1979). The simple scheme of chemical reactivity shown in Fig. 10 is consistent with formation of ester or amide bonds, although it is likely that additional factors involved in the catalytic site, or a relatively loose binding specificity of the protein may influence the selection of the nucleophile with which the electrophilic carbonyl reacts. The difference in susceptibility of the C3 and C4 binding reactions to putrescine and cadaverine discussed above is indicative of such selectivity.

One factor that may be associated with selectivity of this type is the involvement of hydrophobic interactions in the activation and binding. As shown in Fig. 8, the release of bound C3b from Sepharose at alkaline pH is greatly accelerated by a range of detergents. This effect has previously been noted by Law *et al.* (1979*b*) for SDS. The fact that non-denaturing detergents also increase the rate of release of C3b suggests that they may be disrupting a hydrophobic structure in the bound protein, permitting easier access of water to hydrolyse the ester bond. Recent studies by Fontaine *et al.* (1980) demonstrate that both C3 and C3d bind Triton X-100. Thus it is suggested that the reactive centre in native C3 may be in a hydrophobic environment and that conversion of C3 into C3b may, in addition to polarization of the X–C bond (Fig. 10) lead to partial exposure of the reactive group to the aqueous phase. The small effect of ionic strength on C3b binding efficiency (Fig. 7) may be an effect of stabilization of hydrophobic bonding at high ionic strength, leading to decreased binding of C3b. Thus selectivity of the nucleophiles, which can inhibit C3b or C4b binding, or which can at high concentrations inactivate native C3 or C4, may depend partly on their ability to penetrate differing hydrophobic centres in the two proteins. In general, C3 is less sensitive to inactivation by amines than is C4 (Lachmann, 1973, 1979). The inactivation of C3 by KBr or KCNS (Muller-Eberhard *et al.*, 1966), widely used to prepare sera lacking C3 activity, may also be an effect of disruption of hydrophobic bonding, permitting entry of water to the reactive centre, causing inactivation by hydrolysis. Ether and chloroform treatments of sera have also been used to provide reagents deficient in C3 or C4 activity (Harboe *et al.*, 1963; Ratnoff *et al.*, 1954), and these also may act by disruption of a hydrophobic centre.

The scheme shown in Fig. 10 represents a very simple interpretation of present knowledge of C3 covalent-binding activity. It is apparent that C3 and probably also C4 and α_2-macroglobulin possess a type of enzymic active site, capable of catalysing a single 'autoligase' (Twose *et al.*, 1980) or 'autolytic' (Sim & Sim, 1981) event. Although in Fig. 10 activation of C3 is represented as an event that increases the electrophilic properties of a carbonyl group in C3, it is equally possible, on present evidence, that other chemical groupings in the C3 reactive site may enhance selectively the nucleo-

philic properties of suitable binding substrates. Further studies are needed to define the structure of the C3 reactive site.

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