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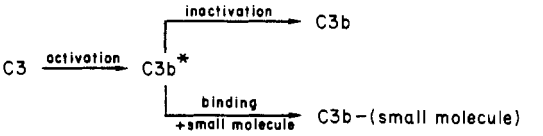
# Binding Reaction between the Third Human Complement Protein and Small Molecules†

Sai-Kit A. Law,* Tana M. Minich, and R. P. Levine

ABSTRACT: The covalent binding reaction of the third complement protein (C3) to receptive surfaces is thought to proceed by the following mechanism. An internal thioester [Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., & Prahl, J. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5764–5768; Law, S. K., Lichtenberg, N. A., & Levine, R. P. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7194–7198], which is usually hidden within the C3 molecule, is exposed upon proteolytic activation of C3 to C3b* (the hypothetical conformation of C3b which has the capacity to bind to receptive surfaces and small molecules). The exposed thioester is accessible to attack by hydroxyl groups on receptive surfaces. An acyl transfer reaction takes place, leading to the binding of C3b to the receptive surfaces via an ester linkage [Law, S. K., Lichtenberg, N. A., & Levine, R. P. (1979) *J. Immunol. 123*, 1388–1394]. We have used a fluid-phase system to demonstrate the specific binding of different small molecules to the labile binding site of C3. The small molecules include glycerol, different hexose monomers, sucrose, raffinose, and four amino acids. These molecules bind to C3b with different efficiencies, indicating that there is an order of preference of C3b* for these molecules. In certain cases, the small molecules bind to C3b via ester linkages (e.g., glucose); in others, the bond is an amide linkage (e.g., lysine). We have also studied the concentration dependence of the binding of small molecules to C3b. The binding is consistent with the following reaction scheme:




The binding of C3b, the activated form of the third complement protein (C3), to cell surfaces is a central step in the humoral immune defense against infection. The binding reaction between C3/C3b and receptive surfaces (RS)[1] has been studied by several laboratories, and a model of the chemistry of the binding reaction has been proposed. This model is schematically shown in Figure 1. There is an internal thioester within C3 (Janatova et al., 1980a; Pangburn & Müller-Eberhard, 1980; Howard, 1980; Tack et al., 1980; Law et al., 1980b) between a cysteinyl residue and a glutamyl residue (Tack et al., 1980). These amino acids are separated by two amino acid residues along the $\alpha$ chain of C3 (Tack et al., 1980). The thioester is probably protected in a hydrophobic pocket since it is quite stable in aqueous medium (Pangburn & Müller-Eberhard, 1980; Law et al., 1980b), and it is more accessible to methylamine than similar nucleophiles of progressively larger sizes such as ethylamine, isopropylamine, and *tert*-butylamine (Pangburn & Müller-Eberhard, 1980). The activation of C3 is initiated by the proteolytic conversion of C3 to C3a and C3b. Activation imparts to the C3b portion of the molecule the capacity to bind to receptive surfaces, and it has been described as the exposure of a labile binding site of C3 (Müller-Eberhard et al., 1966) that has a lifetime on

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[1] Abbreviations: C3b*, the hypothetical conformation of C3b which has the capacity to bind to receptive surfaces and small molecules; RS, receptive surfaces; $CH_3NH_2$, methylamine; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; BE, binding efficiency; $k$, rate of conversion of C3 to C3b* by trypsin; $k_0$, rate of decay of C3b* to C3b in the fluid phase; $k'$, rate of binding of glycerol to C3b* to form C3b–glycerol.

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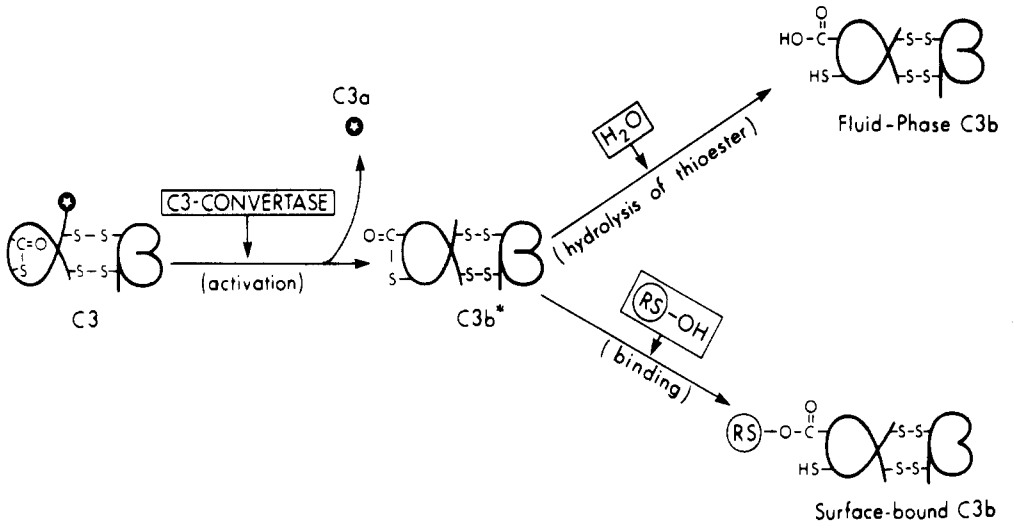



FIGURE 1: Hypothetical binding reaction mechanism of C3 (see text).

the order of a fraction of a second (Götze & Müller-Eberhard, 1970). We will refer to the activated state of C3b as C3b*. It is defined as the conformation(s) of the molecule capable of mediating the binding reaction, and thus it can be visualized as a C3b molecule having an exposed but intact thioester that can either undergo hydrolysis to assume an inactivated state as C3b in the fluid phase or react with hydroxyl groups on nearby RS to form an ester bond (Law & Levine, 1977; Law et al., 1979) probably by an acyl transfer reaction (Law et al., 1979, 1980b; Pangburn & Müller-Eberhard, 1980; Tack et al., 1980; Sim et al., 1981). It should be pointed out that the "exposure" of the thioester is only a description of the functional state of C3b* which may or may not correspond to the actual physical conformation of the molecule.

Though much has been discovered recently about the structure of the labile binding site of native C3, the chemical details of how it participates in the binding reaction are unknown. Transesterification as the binding reaction mechanism is an attractive hypothesis since Tack et al. (1980) have shown the existence of a thioester in native C3 and we have demonstrated that the covalent binding of C3b to RS is by way of an ester bond in the form of C3b–CO–O–RS (Law et al., 1979). The hypothesis lacks the support of the crucial experiment showing that the carbonyl group of the ester between C3b and RS is the same carbonyl group of the thioester in native C3. In addition, the evidence of the binding of C3b to hydroxyl groups is based on the kinetics of the release by hydroxylamine of C3b from the surfaces of zymosan and sheep erythrocyte membranes (Law & Levine, 1977; Law et al., 1979). That the hydroxyl groups to which C3b binds are probably those of sugar residues has been implied from the inhibition of C3b binding to Sepharose–trypsin by different sugar molecules (Capel et al., 1978). Recently, the direct binding of different hydroxyl-containing molecules to the labile binding site of C3/C3b has been reported from several laboratories: Sim et al. (1981) demonstrated the binding of radioactive glycerol and glucosamine to C3b; Mann et al. (1981) were able to bind C3b to the [$^3$H]thioglucose moiety of a specially prepared [$^3$H]glucose–S–S–agarose matrix; Gadd & Reid (1981) showed the binding of C3b to the Fd region of the IgG heavy chain in ovalbumin–antiovalbumin immune aggregates.

We have developed a fluid-phase system which allows us to systematically study the binding reaction of C3b* and different small molecules. In this paper, we report that (i) C3b* binds to sugar molecules as well as amino acids, (ii) the binding efficiencies of different small molecules to C3b* are different and vary over a wide range, (iii) C3b* forms both ester (hydroxylamine-sensitive) and amide (hydroxylamine-resistant) bonds with the small molecules, and (iv) the dependence of the binding reaction on the small molecule concentration is consistent with the reaction scheme as shown in Figure 1.

## Materials and Methods

*Proteins, Reagents, and Chemicals.* Fresh human plasma was purchased from the American Red Cross (St. Louis, MO). Trypsin and soybean trypsin inhibitor (SBTI) were products of Worthington Biochemical Corp. (Freehold, NJ). Various sugars and amino acids and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive molecules were products of New England Nuclear (Boston, MA) except for [$^3$H]fucose, which was purchased from Amersham (Arlington Heights, IL). C3 was isolated and purified from human plasma (Tack & Prahl, 1976), and C3b was prepared from purified C3 according to Tack et al. (1979).

*Binding.* Solutions of radioactive small sugar molecules were prepared by mixing labeled (in ethanol–water) with unlabeled molecules (in water) to a specific radioactivity of 50 mCi/mmol and a concentration of about 10 mM. About 125 $\mu$L of the mixture was delivered to the bottom of a 12 × 75 mm glass tube, and the solvent was evaporated by a stream of nitrogen at room temperature. A 50-$\mu$L aliquot of a C3 solution (10 mg/mL), in 25 mM Veronal buffer containing 0.15 M NaCl, pH 7.5, was added. The final concentration of the small molecule was 25 mM. The mixture was warmed to 37 °C, and 4 $\mu$L of a trypsin solution (1 mg/mL) was added. The reaction was carried out at 37 °C. At the end of 5 min, 0.4 mL of a solution containing SBTI (0.5 mg/mL), PMSF (0.5 mM), and the unlabeled small molecule (0.25 M) was added to stop both the trypsin cleavage of C3 to C3b and the binding of the radioactive small molecule to the labile binding site of C3b. A $NaDodSO_4$ solution (50 $\mu$L) (20% in water) was added to denature the proteins so as to minimize nonspecific binding of radioactive molecules to C3b. The mixture was then dialyzed against 3 × 2 L of 5 mM Veronal buffer, containing 0.15 M NaCl, pH 7.5, followed by 2 L of 5 mM sodium phosphate, pH 7.0, for a total of 18–24 h. The protein concentration and radioactivity were determined. Two control experiments were done; both were identical with the one described above except that one experiment was performed in

Table I: Binding of Small Molecules to C3/C3b*/C3b [a]

| small molecule | binding (nCi/mg of total protein) C3 (+trypsin) | C3 (−trypsin) | C3b | specific binding nCi/mg of total protein | molar ratio of (C3b–small molecule) to (total C3) | $C3\text{–}CH_3NH_2$ (max) | binding efficiency molar ratio of (C3b–small molecule) to (active C3) |
|---|---|---|---|---|---|---|---|
| mannose | 25.8 | 1.6 | 1.7 | 24.2 | 0.126 | 0.62 | 0.20 |
| glucose | 14.5 | 1.9 | 1.8 | 12.7 | 0.066 | 0.52 | 0.13 |
| glucose-2 | 11.3 | 2.2 | 2.1 | 9.2 | 0.048 | 0.42 | 0.11 [b] |
| fucose | 13.0 | 2.9 | 3.8 | 9.6 | 0.050 | 0.47 | 0.11 |
| fructose | 6.5 | 1.1 | 1.3 | 5.3 | 0.028 | 0.35 | 0.08 |
| galactose | 8.3 | 3.1 | 3.0 | 5.2 | 0.027 | 0.42 | 0.06 |
| inositol | 1.5 | 0.4 | 0.6 | 1.0 | 0.011 | 0.52 | 0.02 |
| sucrose | 8.3 | 0.6 | 0.6 | 7.7 | 0.040 | 0.31 | 0.13 |
| raffinose | 32.0 | 8.3 | 10.9 | 22.4 | 0.117 | 0.31 | 0.38 |
| glycerol | 19.6 | 1.3 | 1.3 | 18.3 | 0.095 | 0.35 | 0.27 |
| threonine | 37.6 | 1.4 | 1.2 | 36.3 | 0.189 | 0.58 | 0.33 |
| serine | 21.9 | 3.7 | 3.4 | 18.3 | 0.096 | 0.58 | 0.17 |
| glycine | 12.5 | 2.9 | 3.3 | 9.4 | 0.049 | 0.64 | 0.08 |
| lysine | 24.7 | 17.0 | 18.4 | 7.0 | 0.036 | 0.64 | 0.06 |
| methylamine | 15.1 | 26.1 | 14.1 | ND [c] | ND | 0.62 | ND |

[a] The results shown are data from representative experiments. Each value represents the average of duplicate determinations, which show a variation of about ±5% from the mean. [b] Similar experiments performed at different times may show variations of up to 20% from each other. Data from a second experiment on the binding of C3b to glucose (glucose-2) is included to illustrate this point. [c] ND, not determined.

the absence of trypsin, and in the other, preformed C3b was used in place of C3.

The binding of amino acids to C3b was studied in a similar procedure. Radioactive amino acids (from New England Nuclear, Boston, MA) were supplied in a solution of HCl. The amino acid solution (a mixture of labeled and unlabeled molecules at a specific radioactivity of 50 mCi/mmol) was first neutralized before being dried down in the test tube.

*Determination of C3–$CH_3NH_2$(max).* It is known that C3 loses its activity on storage (Janatova et al., 1980b; Sim et al., 1981). For this reason, it is necessary to determine the C3–$CH_3NH_2$(max) value of the C3 sample in each binding experiment. The C3–$CH_3NH_2$(max) value of a C3 sample is the maximum amount of methylamine that can be incorporated into the protein, and the molar ratio of methylamine to C3 represents the fraction of active C3 in the sample (Law et al., 1980b).

The procedure used to determine C3–$CH_3NH_2$(max) was similar to the binding experiment in the absence of trypsin. [$^{14}$C]Methylamine (about 5 mCi/mmol) was used at a concentration of 10 mM, and the incubation was carried out at 37 °C, pH 8.0, for 3 h. Excess methylamine was removed by dialysis. Nonspecific binding of [$^{14}$C]methylamine to C3 was estimated by the binding of [$^{14}$C]methylamine to preformed C3b under identical incubation conditions.

*Calculations.* The binding efficiency of a small molecule to C3b is defined as the molar ratio of (C3b bound to small molecules)/(potentially active C3), which can be expressed as (C3b bound to small molecules)/[(total C3)[C3–$CH_3NH_2$(max)]]. C3–$CH_3NH_2$(max) is determined as described in the previous section, and the molar ratio of (C3b bound to small molecules)/(total C3) is given by $AB/(CD)$ where $A$ = the radioactivity incorporated per unit weight of protein (in millicuries per milligram, $B$ = the molecular weight of C3, which is taken to be 185 000, $C$ = the ratio of C3 to total protein by weight, which is C3/(C3 + trypsin + SBTI) and is numerically equal to 0.71 in these experiments, and $D$ = the specific radioactivity of the small molecule used, which is 50 mCi/mmol (or 25 mCi/mmol in the case of inositol).

*Hydroxylamine Treatment of C3b–(Small Molecule) Complexes.* C3b–(small molecule) complexes, in 2% Na-$DodSO_4$, were dialyzed into 5 mM sodium phosphate, pH 7.0, at room temperature. An equal volume of hydroxylamine (2 M) in 0.2 M $NaHCO_3$, pH 9.0, was added. The mixture was incubated at 37 °C for 1 h. Hydroxylamine, as well as released small molecules, was removed by dialysis against 3 × 2 L of 5 mM sodium phosphate, pH 7.0, for a total of 18–24 h. The small molecules were used at 250 mCi/mmol in the preparation of C3b–(small molecule) complexes in these experiments.

*$NaDodSO_4$–Polyacrylamide Gel Electrophoresis.* Na-$DodSO_4$–polyacrylamide gel electrophoresis was carried out in the Laemmli system (Laemmli, 1970) under reducing conditions with slab gels having a polyacrylamide gradient of 6–12%. The gels were stained and destained according to Fairbanks et al. (1971).

*Other Assays.* Protein concentrations were determined by the method of Lowry et al. (1951). C3 hemolytic activity was measured as described (Law et al., 1980b). Gel slices containing radioactively labeled polypeptides were solubilized by incubation in a solution of 4% Protosol in Econofluor (products of New England Nuclear, Boston, MA) at 37 °C for 18–24 h before counting. The counting efficiency for $^3$H is 39%, and that for $^{14}$C is 81%.

## Results and Discussion

*Binding of Small Molecules to C3/C3b.* Small molecules (25 mM) were incubated with C3 (10 mg/mL) in the presence of trypsin (80 μg/mL) which served as the C3 convertase in these experiments. The reactions were carried out in 25 mM Veronal buffer containing 0.15 M NaCl, pH 7.5, at 37 °C for 5 min. The results are shown in Table I. The binding efficiency of a small molecule is defined as the fraction of active C3 specifically labeled at the labile binding site when it is activated by trypsin to C3b* under the conditions specified. The measurement is only valid when two criteria are met: (i) the reaction is complete; and (ii) the binding is specific.

The binding reaction is complete when all the C3 molecules are converted to C3b* and all the C3b* molecules either decay to inactive C3b or react with the small molecules to form C3b–(small molecule) complexes. The half-life of C3b*, which was estimated to be on the order of a fraction of a second (Götze & Müller-Eberhard, 1970), is short in comparison with the time for the proteolytic conversion of C3 to C3b*. In the

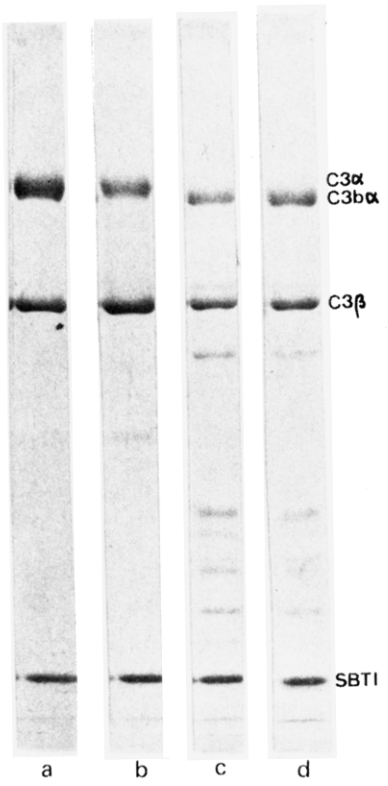



FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of C3 and C3b samples: (a) C3 incubated with [$^{14}$C]methylamine; (b) C3 incubated with [$^3$H]threonine in the absence of trypsin; (c) preformed C3b incubated with [$^3$H]threonine; (d) C3 incubated with [$^3$H]-threonine in the presence of trypsin. Some C3 degradation products are shown as minor bands in (c) and (d). SBTI, which was added to the samples to stop trypsin activity, is present in all samples. The Coomassie Blue stained gel is shown here.

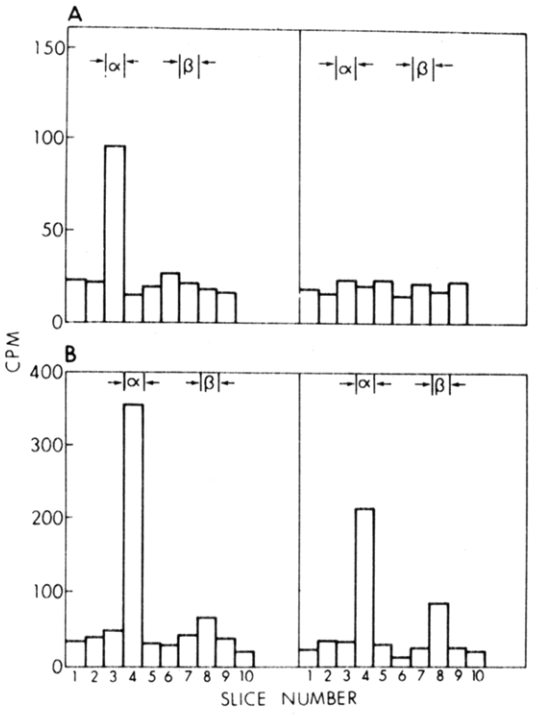



FIGURE 3: Binding of [$^3$H]glucose (A) and [$^3$H]lysine (B) to C3 in the presence (left panels) and absence (right panels) of trypsin. The radioactivity associated with the $\alpha$- and $\beta$-polypeptides of C3/C3b is shown.

experiments presented in Table I, the conversion of C3 to C3b*, detected as C3b, occurs on the order of 1 min. It is therefore justified to use the conversion of C3 to C3b as a means to determine the completeness of the binding reaction. We analyzed the reaction mixture of each experiment by $NaDodSO_4$–polyacrylamide gel electrophoresis: a total disappearance of the C3$\alpha$ polypeptide in conjunction with the appearance of the C3b$\alpha$ polypeptide indicates a complete reaction. A typical gel is shown in Figure 2.

In order to obtain an accurate value for the binding efficiency, the amount of nonspecific binding of the small molecule to C3/C3b must be determined. Two different kinds of nonspecific binding have to be considered in these experiments: one is the covalent incorporation of the radioactive molecules to proteins in general, and in this case, C3/C3b; the other is the binding of the radioactive molecules to the labile binding site of C3 in the absence of trypsin. The covalent incorporation of radioactive molecules to proteins is an inherent problem with commercially obtained radioactive compounds. It has been shown that $^3$H- and $^{14}$C-labeled glucose binds to different proteins upon incubation at 37 °C and that the degree of binding varies with different batches of radioactive glucose from different manufacturers (Trüeb et al., 1980). This type of nonspecific binding is determined by the incubation of the small molecules with preformed C3b.

The binding of small molecules to the labile binding site of C3 in the absence of trypsin creates another problem in the study of the binding reaction. One of these molecules is methylamine, which binds and inactivates C3 (Pangburn & Müller-Eberhard, 1980; Howard, 1980; Tack et al., 1980; Law et al., 1980b). The degree of binding directly correlates with the degree of inactivation, and the reaction proceeds at an appreciable rate (Law et al., 1980b). If we incubate C3 with methylamine in the presence of trypsin, the two forms of binding, the binding of methylamine to both native C3 and C3b*, occur simultaneously, with a continuous conversion of C3 to C3b* by trypsin. The binding of methylamine to C3b* is therefore difficult to determine. For the reason that other known inactivators of C3, e.g., ethylamine and isopropylamine (Pangburn & Müller-Eberhard, 1980), might bind to the labile bindng site of native C3 in the same manner as methylamine, their reaction with C3b* was not studied. Indeed, there is a need to establish that the small molecules that we studied do not bind to the labile binding site of native C3 in the absence of trypsin, at least not at a significant level within the 5-min reaction period. We therefore did the control experiment by incubating the small molecules with C3 in the absence of trypsin. Only when the amount of binding of the small molecules to native C3 is not significantly different from their binding to preformed C3b would we include the data in Table I. The values for a methylamine binding experiment, which is performed exactly like that for other small molecules, are also shown in the table to illustrate this point.

*Binding of Small Molecules to the $\alpha$ Chain of C3b.* To demonstrate the specific binding of small molecules to the labile binding site of C3/C3b, we show the specific binding is to the C3b$\alpha$ polypeptide. The results of the experiments with [$^3$H]glucose and [$^3$H]lysine are shown in Figure 3. The binding of [$^3$H]glucose to C3/C3b in the presence of trypsin is shown to be exclusively to the $\alpha$ chain of C3b (Figure 3A, left panel). No detectable counts were found in association with the C3$\beta$ polypeptide or with either the $\alpha$ or the $\beta$ chains of C3 in the absence of trypsin (Figure 3A, right panel). [$^3$H]Lysine, which has a high nonspecific binding to C3 in the absence of trypsin (see Table I), binds to both C3$\alpha$ and C3$\beta$ polypeptides (Figure 3B, right panel). However, significant enhancement of binding to the C3b$\alpha$ polypeptide is demonstrated when [$^3$H]lysine is incubated with C3 in the presence

Table II: Hydroxylamine Release of Small Molecules from C3b-(Small Molecules) at pH 9

| small molecule | specific radioactivity of C3b-(small molecule) (nCi/mg) | specific radioactivity and % radioactivity retained after incubation at pH 9 | | | | tentative bond between C3b and small molecule |
|---|---|---|---|---|---|---|
| | | −hydroxylamine | | +hydroxylamine | | |
| | | nCi/mg | % | nCi/mg | % | |
| glucose | 75.7 | 64.5 | 85 | 2.9 | 4 | ester |
| glycerol | 124.3 | 102.1 | 82 | 15.7 | 15 | ester |
| lysine | 53.5 | 38.2 | 71 | 33.1 | 62 | amide |
| serine | 95.8 | 81.3 | 85 | 78.1 | 82 | amide |
| threonine | 344.8 | 327.3 | 95 | 300.8 | 87 | amide |

of trypsin (Figure 3B, left panel). The specific binding of serine and threonine to the C3b$\alpha$ polypeptide was also demonstrated in similar experiments (data not shown).

*Nonspecific Binding of Glucose and Lysine to C3.* In instances where it was found that a given small molecule bound to C3 (in the absence of trypsin) and preformed C3b with similar efficiency, we proceeded to determine its binding efficiency to C3b*. We studied in detail the nonspecific binding of [$^3$H]glucose and [$^3$H]lysine to C3. Glucose was chosen as representative of a hydroxyl-containing molecule and lysine as representative of an amine-containing molecule. C3 was incubated with [$^3$H]glucose or [$^3$H]lysine (25 mM, 50 mCi/mmol) at pH 7.5, 37 °C, for 0, 1, 2, and 3.4 h. Unbound [$^3$H]glucose or [$^3$H]lysine was removed by dialysis. The amount of [$^3$H]glucose or [$^3$H]lysine bound to C3, along with the residual hemolytic activity of C3, was determined. The results are shown in Figure 4. The incorporation of [$^3$H]-glucose and [$^3$H]lysine to C3 increases with time, but the binding does not affect the hemolytic activity of C3. We therefore argue that this form of binding of [$^3$H]glucose and [$^3$H]lysine to C3, even for an extended period of incubation time, does not involve the labile binding site.

*Ester or Amide Bond between C3b and Small Molecules.* In light of our current understanding of the chemistry of the labile binding site of C3, an earlier proposal that the binding of C3b to RS takes the form of C3b–CO–O–RS must be extended to encompass amide bond formation. Other nucleophilic groups, such as amines, should also be able to react with the exposed thioester of C3b* to form amide bonds which would be resistant to hydroxylamine treatment. In fact, it has been demonstrated that the binding of C3b to immune aggregates is by way of both hydroxylamine-sensitive and hydroxylamine-resistant bonds (Gadd & Reid, 1981). C4/C4b, which has similar molecular and binding properties to C3/C3b (Law et al., 1980a,b; Gorski & Howard, 1980; Campbell et al., 1980; Janatova & Tack, 1981), also binds to immune aggregates via the two types of linkages (Campbell et al., 1980). It is possible, therefore, that C3b can bind covalently to RS by way of hydroxylamine-resistant as well as hydroxylamine-sensitive bonds and that the binding of C3b to sheep erythrocyte membranes (Law & Levine, 1977), zymosan (Law et al., 1979), and Sepharose (Sim et al., 1981) by ester linkages is merely a reflection on the abundance of hydroxyl groups, in comparison with amino groups, on these surfaces.

We have demonstrated the specific binding of lysine and glycine to C3b* (see Table I). The C3b–lysine complex is stable in hydroxylamine since no significant amount of hydroxylamine-specific release of lysine from the C3b–lysine complex was detected (Table II). We tentatively conclude that C3b* can react with the amino group(s) of lysine and that the C3b–lysine complex is probably of the form C3b–CO–NH–lysine.

The stability of the C3b–glucose, C3b–glycerol, C3b–serine, and C3b–threonine complexes in alkaline hydroxylamine was

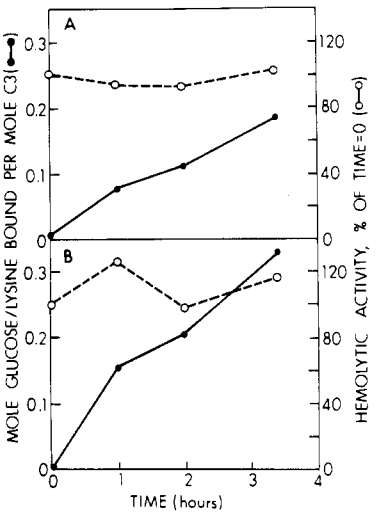



FIGURE 4: Binding of [$^3$H]glucose (A) and [$^3$H]lysine (B) to C3 in the absence of trypsin as a function of time of incubation (●). The residual hemolytic activity of the C3 sample after incubation (O) is expressed as a percentage of the sample incubated for zero time.

also studied (Table II). Glycerol and glucose, which contain hydroxyl groups, bind to C3b via hydroxylamine-sensitive bonds. Treatment with the nucleophile at pH 9 releases these small molecules from their covalent complexes with C3b. Serine and threonine, however, were not released from their complexes under identical conditions, indicating that C3b probably binds to the amino acids via their $\alpha$-amino groups. One explanation of this observation is that the hydroxyl groups of the amino acids are not reactive to C3b*. It is also possible that C3b* reacts with the hydroxyl groups of the amino acids to form C3b–CO–O–(amino acid) complexes initially. The C3b–CO–O–(amino acid) complexes then undergo an intramolecular O to N acyl shift from the $\beta$-hydroxyl group to the $\alpha$-amino group of the amino acids (Erickson & Merrifield, 1976) to attain the C3b–CO–NH–(amino acid) forms. The rearrangement reaction occurs quite readily, in the order of minutes to completion, at neutral pH (Erickson & Merrifield, 1976). Thus, only the hydroxylamine-resistant forms of the C3b–serine and C3b–threonine complexes were observed.

*Efficiency of Binding of Small Molecules to C3b**. It is evident from Table I that C3b* can react with different forms of carbohydrates, from glycerol to different hexoses, sucrose, and raffinose, as well as amino acids. However, C3b* is selective in its reaction with different small molecules in the sense that there is an order of preference for certain structures, e.g., raffinose (BE = 0.38) vs. glucose (BE = 0.12). We have also performed inhibition experiments to confirm the relative order of preference of C3b* for different small molecules. For example, we have used unlabeled mannose and glucose to inhibit the binding reaction between C3b* and [$^3$H]glucose and have consistently found that mannose is a more potent inhibitor than glucose, which is in agreement with the data

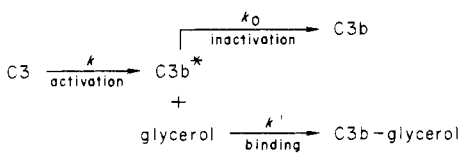
(Table I) that mannose has a higher efficiency of binding to C3b* than glucose. (Details of these experiments will be presented in a separate paper.)

The selectivity of the reaction between C3b* and different small molecules suggests that the reaction is not a simple acyl transfer of the carbonyl group of the thioester to the hydroxyl or amino groups of the small molecules, since the mechanism of a reaction of this sort predicts no preference in reactivity of C3b* with molecules as similar as mannose, glucose, and galactose. The efficiency of binding of C3b* to these three aldohexose isomers differs markedly: binding to mannose is close to 2 times more efficient than that to glucose, and the efficiency of binding to glucose is about twice that to galactose. From these data, we deduce that some molecular feature(s) or C3b* in a spatial or chemical sense, must be responsible for the selectivity.

We can also argue that C3b* probably reacts with different efficiencies with different groups within the same molecule although we do not have data to support this argument. From the data presented, we cannot tell whether or not both amino groups of lysine can react with C3b*; neither can we demonstrate that the hydroxyl groups of serine and threonine react with C3b*. Furthermore, we cannot demonstrate that all of the hydroxyl groups of a carbohydrate molecule, e.g., glycerol, are equally reactive. We plan to study the binding reaction between C3b* and derivatives of the small molecules, methylated at appropriate groups. Since the methylated small molecules are not easily available in radioactive form, we will study their inhibitory effect on the binding reaction between C3b* and radioactive small molecules, e.g., [$^3$H]glycerol. These experiments are currently in progress.

*Fluid-Phase System.* The fluid-phase system we describe in this paper will be most useful to study the binding reaction of C3b*. We can specifically label the C3b* molecule at the labile binding site with radioactive small molecules of high binding efficiencies. Labeled C3b can be generated in quantity, and it is possible to locate the amino acid on C3 through which C3b* reacts with small molecules. If the binding occurs by a transesterification mechanism (see Figure 1), it is expected that the labeled small molecule will bind to the same glutamyl residue that is labeled by the methylamine treatment of native C3 (Tack et al., 1980).

Kinetics studies of the binding reaction are also possible in the fluid-phase system. The system is simple and consists of only three components: C3; a small molecule of choice; and trypsin, which serves as the C3 convertase. Thus, we can avoid the uncertainties of unknown components on cell and other receptive surfaces as well as the complicated kinetics of the natural C3 convertases, which are quite labile. The system also has the advantage of being in the fluid phase, where all components are homogeneous in solution and free diffusion is defined. We have used this system to study the binding reaction between C3b* and glycerol at different glycerol concentrations. The result (Figure 5) is in agreement with the hypothesis that the binding reaction undergoes the following scheme:




with $k$ as the rate of conversion of C3 to C3b*, $k_0$ the rate of decay of C3b* to C3b in the fluid phase, and $k'$ the rate of binding of C3b* to glycerol. This scheme predicts that the dependence of the binding efficiency (BE) as a function of glycerol concentration follows

$$\frac{1}{BE} = 1 + \frac{k_0}{k'}\frac{1}{[\text{glycerol}]}$$

(The theoretical derivation of this equation will be presented in a separate paper.) A plot of the inverse of the binding efficiency against the inverse of glycerol concentration should give a straight line intersecting the $y$ axis at 1. The slope of the straight line would represent the ratio of $k_0/k'$, the relative reaction rate of C3b* with water and glycerol. As shown in Figure 5, we indeed obtain a straight line on plotting $(BE)^{-1}$ vs. $[\text{glycerol}]^{-1}$ from experimental data. Moreover, the straight line intersects the $y$ axis at about 1, and the slope, representing $k_0/k'$, is calculated to be 0.066 M. A similar plot is obtained from another experiment in which glucose was used as the binding small molecule instead of glycerol.

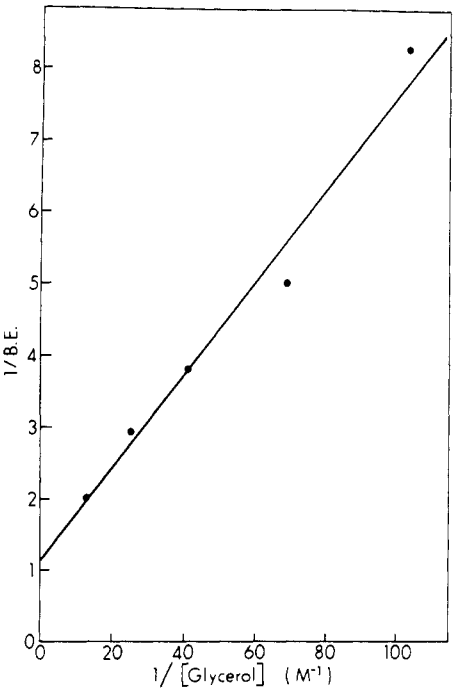



FIGURE 5: Binding of [$^3$H]glycerol to C3b* as a function of glycerol concentration.

We are currently employing this fluid-phase system to further study the binding reaction of C3b* to different molecules. We will be using a similar system in an attempt to bind small molecules to the labile binding site of C4b. If such binding is demonstrated, the comparison of the specifics between the binding reaction of C3/C3b*/C3b and that of C4/C4b*/C4b should yield information that will aid us in understanding the labile binding sites of these molecules.

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# Interaction of Saccharide Haptens with Myeloma Proteins. A 270-MHz Proton Nuclear Magnetic Resonance Study†

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ABSTRACT: 270-MHz proton NMR is used to study U61, E109, and J539, three homogeneous IgA mouse myeloma proteins which have specificities for saccharides. Three histidine resonances are observed to titrate in U61 and E109 and two in J539. These two as judged by their p$K_a$ values (4.8 and 7.4), titration range, and chemical shifts are common to all three proteins. On the basis of work on light chains, these are assigned to His-198$_L$ (p$K_a$ = 4.8) and His-189$_L$ (p$K_a$ = 7.4). Neither of these is perturbed on hapten binding. The third histidine (p$K_a$ = 6.7) common in both U61 and E109 is assigned to His-53$_H$ or His-58$_H$, both in the hypervariable region. This histidine is perturbed slightly on hapten binding in U61. Difference spectra show that comparatively few protein resonances are perturbed on binding hapten, suggesting that accompanying conformational changes are limited to the combining site. The difference spectra of U61 and E109 on addition of inulotriose suggest very similar binding sites which contain aromatic residues. Difference spectra of J539 binding to the mono-, di-, and trigalactosides suggest that the first subsite is nonaromatic, unlike the second and possibly the third. The binding of *p*-nitrophenyl β-D-galactopyranoside to J539 is consistent with a second aromatic subsite. The exchange rate for this hapten between J539 and the free solution is 2545 $s^{-1}$, and the chemical shifts of the anomeric proton and those of the H(3) and H(6) protons of the nitrophenyl ring are all upfield. *m*-Nitrophenyl acetate binds weakly to J539. It is suggested that the importance of aromatic residues in binding sites for D sugars arises in part from the sugars adopting a $^4C_1$ D conformation in which there is a planar hydrophobic face which can interact with planar rings on aromatic side chains. The specificity for sugar antigens is provided by having many subsites which favor polymeric carbohydrate antigens and discriminate against smaller hydrophobic ligands which bind to an individual subsite but with relatively low affinity.

Saccharides form a widely distributed and very important class of naturally occurring antigens. As well as being encountered in food and in the environment, polysaccharides are major constituents of bacterial cell walls and provide the antigenic determinants responsible for the differentiation of blood groups. This importance is reflected in the large number of homogeneous mouse myeloma proteins found to have specificity for saccharide ligands (Glaudemans, 1975; Glaudemans et al., 1975).

Although polysaccharide antigens with molecular weights of several million exist, the region of the macromolecule recognized by the combining site is still very restricted. A hexasaccharide of alternating D-glucose and D-glucuronic acid residues has been postulated as the determinant in *S. pneumoniae* type III polysaccharide (Mage & Kabat, 1963). Determinants as small as disaccharides may exist (Kabat, 1968). There is, however, considerable scope for antigenic

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