THE SUBUNITS IN RABBIT ANTI-FORSSMAN IGM ANTIBODY

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Several authors have recently studied the number and nature of the subunits obtained by reduction of IgM and, when the IgM was a specific antibody, have compared the number of antigen-combining sites on the original molecule and on the subunits. Onoue, Yagi, Grossberg, and Pressman (1) reported that rabbit IgM antihapten antibody had about six binding sites, and that on reduction with 0.1 M 2-mercaptoethanol and subsequent alkylation, subunits were obtained with sedimentation coefficients about 6S, and the same total number of binding sites per unit weight as in the parent molecule. Hill and Cebra (2) reduced horse IgM antibody against Type I pneumococcus polysaccharide, and obtained 6.3S subunits which no longer precipitated with antigen, but which retained virtually all the capacity of the intact antibody to bind antigen when an indirect precipitation method was used. Since Miller and Metzger (3), and Lamm and Small (4) have reported that human and rabbit IgM respectively have 10 heavy (H) and 10 light (L) chains, the numbers of subunits and of binding sites are presumably multiples of five. Miller and Metzger reported that the subunits of an IgM paraprotein are structurally similar to IgG and are composed of two H and two L chains. They suggest that 10 potential antigen-binding sites would be expected on each IgM molecule. Suzuki and Deutsch (5, 6) however have presented evidence that human IgM paraproteins comprise 10 H and 15 L chains, and that mild reduction without alkylation gives rise to subunits composed of two H and three L chains, with sedimentation coefficient 8S. According to these authors, the 8S subunits after alkylation of $-SH$ groups lose two L chains and are converted to 7S components comprising two H and one L chain associated together.

In contrast to the reports quoted above, using purified rabbit anti-Forssman IgM, isolated by two quite different methods, we have obtained on mild reduction subunits which appear to be composed of a single H and L chain. Furthermore only half of these subunits could be shown to have specific binding sites for Forssman antigen. Our experiments involved working at very low concentrations of antibody, radiolabeled by one or other of two separate techniques, and detecting antigen-binding sites by means of antigen columns. Although rabbit anti-Forssman IgM is a highly avid antibody (7, 8) the subunits proved to be of low avidity, and their binding sites could be adequately demonstrated only by a sensitive method of this kind.

Materials and Methods

Preparation of Antisera.—Healthy Sandy Lop rabbits reared at the National Institute for Medical Research, Mill Hill, London, and New Zealand white rabbits reared at the National Institutes of Health, Bethesda, were used. Anti-Forssman antibody was prepared by repeated intravenous injection of rabbits with boiled sheep stroma over a period of 2½ wk. Blood was taken on the 4th and 5th day following the last injection of antigen and allowed to clot. Serum was titrated for hemolysin activity by the method of Rapp (9) and stored frozen at −20°C. Sera selected for use had a hemolysin titer greater than 40,000 Ab_{50} (i.e. a dilution of 1/40,000 was sufficient to lyse one-half of a population of 2.5×10^8 sheep cells in 15 min in the presence of a 1:7 dilution of guinea pig complement). Individual bleedings from a single rabbit were used for most experiments, but similar results were obtained with pooled sera.

Reagents Used.—Cleland's reagent (dithiothreitol) was obtained from Cyclo Chemical Co. (Los Angeles, Calif.). Carrier-free ^{125}I and ^{131}I with no reducing agent were obtained from The

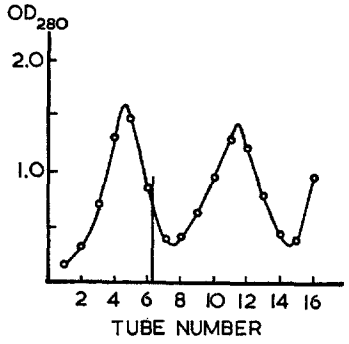


FIG. 1. Ultracentrifugation pattern in a 10–30% sucrose gradient of the pooled first peak and first portion of the second peak separated on Sephadex G-200 from hyperimmune rabbit antiserum. Sedimentation at 25,000 rpm for 15 hr in a Spinco model L ultracentrifuge with a 40 rotor.

Radiochemical Centre, Amersham, England. Iodoacetamide-^{14}C (specific activity 1.23 mc/mM) was obtained from the New England Nuclear Corp. (Boston, Mass.). Before use 5 mg of iodoacetamide-^{14}C were mixed with 25 mg of unlabeled twice recrystallized iodoacetamide and the mixture was recrystallized conveniently and with minimal loss of material from toluene:ethanol (19:1).

General Techniques.—Radioiodine counting was performed in a Packard autogamma well-type scintillation counter under standardized conditions. Background was below 18 cpm. ^{14}C counting was performed in a Packard Tricarb liquid scintillation counter model 4312. 10 volumes of NCS Reagent (Nuclear Chicago Corp., Des Plaines, Ill.) were added to 0.1 or 0.2 ml of sample. To 1.1 or 2.2 ml of the clear mixture were added respectively 10 or 15 ml of toluene containing 5 g of PPO and 0.5 g POPOP per 1. Backgrounds of 13 and 18 cpm respectively were subtracted. No selective quenching was found.

Density gradient ultracentrifugation was performed in a model L Spinco with a 40 rotor. Linear gradients were prepared from solutions of sucrose in saline buffered with veronal at pH 7.6 ionic strength 0.15 (VBS), the concentrations of sucrose being expressed as grams per 100 ml. Samples containing 20–100 μg protein were applied. Sedimentation was performed for 15 hr at 13°C. Gradients of 5–20% sucrose were centrifuged at 35,000 rpm, and gradients

of 10–30% sucrose at 25,000 rpm. At the end of the run 15–20 equal fractions were obtained after puncturing the bottom of the tube (10).

Preparations of Specific IgM Antibody and Its Subunits.—

Method 1: In one series of experiments the globulin fraction from 10–20 ml of rabbit serum was obtained by precipitation in 5.4 M cold ammonium sulfate. The slurry of globulin precipitate was applied to a column of Sephadex G-200 (45 x 600 mm) which was eluted with VBS and 5 ml fractions were collected (11). Fractions from the first protein peak containing the bulk of the IgM and the first portion of the second peak were pooled and concentrated with a Diaflo® apparatus (Amicon Corp., Lexington, Mass.). The concentrate was sedimented through a 10–30% sucrose density gradient (Fig. 1). Fractions containing the bulk of the IgM were pooled. To 21.5 ml of the pool (hemolytic titer 31,000) was added 2.3 ml of 0.1 M EDTA pH 7.6 followed by 5 mg of highly purified water-insoluble[1] Forssman hapten (kindly supplied by

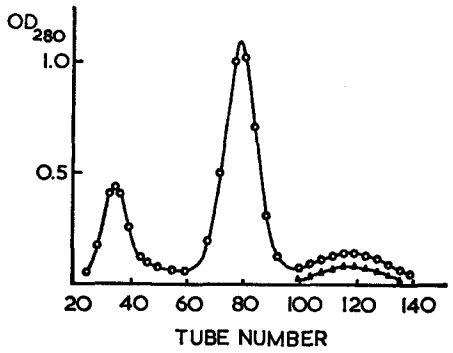


FIG. 2. Separation on Sephadex G-200 in VBS of the concentrated eluate from formolized sheep erythrocytes complexed with Forssman antibody and treated at pH 10.6 at 0°C for 10 min. Brown material which absorbed at 310 mμ and cross-reacted with albumin was found in the third peak. o———o, OD_{280}; and ▲———▲, OD_{310}.

H. J. Rapp) in 0.29 ml absolute alcohol (11). The quantity of Forssman hapten was varied in different experiments so that 60–90% of the hemolysin was absorbed. The mixture was incubated for 1 hr at 37°C and the antigen-antibody complex was sedimented at 1000 *g* for 15 min at 4°C. The heavy precipitate was washed four times in VBS and then resuspended in 2 ml of 0.2 M NaCl with 0.05 M Tris pH 8.6. To this was added 2 ml of freshly prepared 0.006 M dithiothreitol and the mixture was incubated with mixing for 1 hr at 30°C. It was then chilled in an ice bath and recrystallized iodoacetamide-^{14}C added to obtain a final concentration of 0.005 M in some and 0.008 M in other experiments. The subsequent behavior of the material was similar at either concentration. After 1 hr at 0°C the mixture was centrifuged. The bulk of the ^{14}C-labeled protein was found in the supernatant fluid. The ^{14}C-labeled antibody that remained attached to antigen was now displaced by resuspending the complex in successive 1 ml aliquots of intact unlabeled IgM antibody. The mixture was incubated at either 37° or 56°C for 1 hr and recentrifuged to obtain the supernatant fluid. The resulting ^{14}C-labeled reduced and alkylated antibody solutions were pooled and dialyzed. It should be mentioned that dithiothreitol was shown not to affect the antibody-binding capacity of Forssman antigen on erythrocytes under the conditions used.

[1] The water-insoluble Forssman material was prepared as described in reference 11 but was used prior to the final phenol extraction which confers water solubility.

Method 2: In a second series of experiments to be reported in detail elsewhere, intact anti-Forssman antibody was prepared by elution of antibody from formolized sheep erythrocytes. Well washed formolized erythrocytes (12) were mixed with anti-Forssman serum in a quantity sufficient to remove 80–90% of the hemolytic antibody. (These conditions were designed to ensure preferential recovery of IgM antibody and to minimize nonspecific attachment of other immunoglobulins.) They were washed 8–10 times with cold VBS, resuspended in saline as a 30–50% suspension, and chilled in ice. Cold N/10 NaOH was added dropwise until the pH was 10.6. The mixture was held at this pH for 10 min, and then centrifuged briefly at 0°C. The supernatant fluid was immediately adjusted to pH 7.0. As measured by the hemolytic activity 25–50% of the antibody complexed to the cells was recovered in the eluates. These were concentrated by pressure dialysis and passed through a column of Sephadex G-200 (Fig.

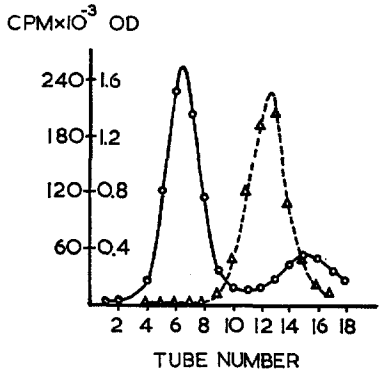


FIG. 3. Ultracentrifugation pattern in a 10–30% sucrose gradient of the material eluted in the first peak from Sephadex G-200 (Fig. 2) labeled with ^{125}I and material from the second peak labeled with ^{131}I. O———O, ^{125}I; and Δ———Δ, ^{131}I.

(The tail on the ^{125}I macroglobulin is due to a trace of albumin, which is commonly found associated with IgM prepared by gel filtration and which is preferentially labeled during iodination. When the labeled IgM is resedimented through a similar sucrose gradient no tail is present.)

2). The macroglobulin peak was further concentrated and 0.5–1.0 mg was labeled with ^{125}I by the iodine monochloride method of McFarlane (13) at mean levels in different experiments of 9–20 atoms of I/mole (assumed mol wt. 900,000). Preliminary experiments had indicated that at least 35 atoms of I/mole can be introduced without detectably affecting the hemolytic activity, and we aimed to introduce sufficient iodine for the radiolabel to be present on both heavy and light chains. The specific radioactivities were about 250 μc/mg of antibody. No loss of hemolytic activity or agglutinin titer was detected. The iodinated IgM mixed with an equal quantity of the same unlabeled IgM was then further purified by sedimentation in a 10–30% sucrose gradient (Fig. 3). 85% of the radioactivity of the ^{125}I bound specifically to sheep erythrocytes, while less than 2% would bind to formolized human or to normal rabbit erythrocytes. The ^{125}I-labeled IgM was checked for the presence of contaminating IgG and IgA by diluting samples of this material in normal rabbit serum and performing a radioimmunodiffusion study with specific anti-rabbit γ-chain and anti-rabbit α-chain antibodies as the developing antiserum.[2] Less than 2% contamination was found.

[2] The antisera were kindly supplied by Dr. S. Utsumi and Dr. J. Cebra respectively.

A solution of the radiolabeled antibody was reduced with 0.002 M Cleland's reagent in 0.2 M NaCl and 0.05 M Tris pH 8.6 for 1 hr at room temperature and alkylated at 0°C with 0.003 M iodoacetamide.

Separation of H and L Chains.—H and L chains were separated on 10 x 700 mm columns of Sephadex G-100 using either 0.04 M sodium dodecyl sulfate (SDS) or 6 M guanidine as solvent. Fractions of 1 ml were obtained.

Dectection of the Presence of H and L Chains.—The amounts of radio-labeled subunits which emerged unretarded from the antigen columns were too small to permit separation of H and L

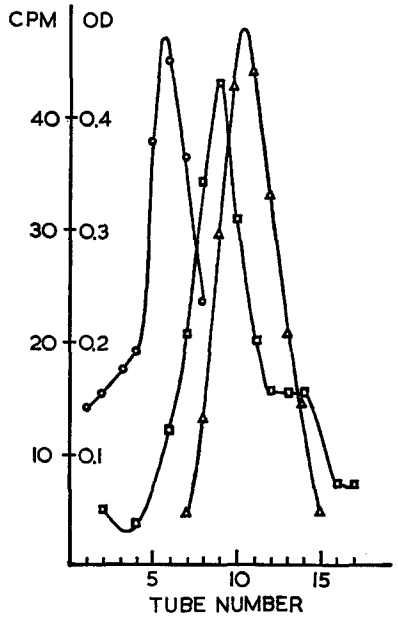


FIG. 4. Ultracentrifugation pattern in a 5–20% sucrose gradient of anti-Forssman IgM reduced and labeled with iodoacetamide-^{14}C (□———□), human IgG (○———○) and hemoglobin (△———△). Sedimentation at 35,000 rpm for 15 hr in a Spinco Model L with a 40 rotor. The OD of the IgG was measured at 280 mμ and of the hemoglobin at 412 mμ.

chains by the above method. The presence of these chains was therefore examined by a quantitative complement fixation technique (C.F.T.) (14) scaled down for use with 1/60th of the usual amounts of reagents. Sheep antisera against rabbit light chains and against rabbit μ-chains were kindly provided by Dr. S. Sell and Dr. T. Borsos respectively. When tested by double diffusion in agar gel against rabbit IgG and reduced and alkylated IgM, the anti-L chain serum was monospecific and showed a reaction of complete identity between the two; the anti-μ-chain serum reacted almost exclusively with IgM, but also contained a trace of anti-L chain which could not be removed by absorption with IgG without rendering the serum anticomplementary. The antisera were used diluted 1:40 or 1:80.

Antigen Columns.—Two types of antigen column were used. The first consisted of diatomaceous earth (Celite) mixed with the cell membranes from 12 ml of packed sheep erythrocytes, prepared by lysis with water followed by very extensive washing with VBS. Such

columns had very slow flow rates. Much better flow rates were obtained by mixing boiled stroma equivalent to 30 ml of packed erythrocytes with 10 g of cellulose (Hyflo Super-Cel, Johns-Manville, N.Y.C.) and packing into 12 × 300 columns. The columns were pretreated to prevent nonspecific binding of protein by passing through them normal rabbit serum which had been absorbed with 1/10th volume of formolized sheep erythrocytes. 0.05 part of absorbed normal rabbit serum was also added to the VBS used for elution, except in those experiments designed to demonstrate whether H and L chains were both present in the antibody material eluted from the columns without retardation. For such experiments the columns were first

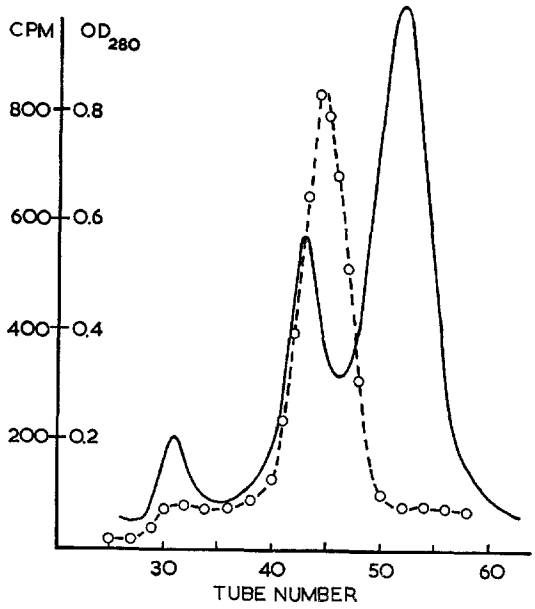


FIG. 5. Elution pattern from Sephadex G-200 of reduced and alkylated ^{125}I-labeled anti-Forssman IgM mixed with normal rabbit serum. O - - - O, radioactivity of IgM subunits; and ———, OD_{280}.

flushed continuously with VBS for 2 wk, until the effluent buffer gave no detectable C.F.T. with the antisera to L or μ-chains. This procedure did not result in nonspecific protein binding.

To the columns were applied 0.4–1.0 ml containing 100–250 μg of ^{14}C-labeled or 5-100 μg of ^{125}I-labeled preparations. They were eluted in fractions of 0.5–1.0 ml. Proteins which were not expected to complex with Forssman antigen were used as markers. These included hemoglobin, rhodamine-labeled BSA and, in a few experiments, normal serum IgM labeled with ^{131}I, which had been reduced and alkylated after prior removal of any antibody to erythrocytes by absorption.

RESULTS

Size and Nature of the IgM Subunits.—Fig. 4. shows the sedimentation pattern in a 5–20% sucrose gradient of the ^{14}C-labeled subunit released after reduction and alkylation of antibody complexed with Forssman hapten. A human

IgG marker and hemoglobin marker were mixed with the sample prior to centrifugation. The reduced and alkylated material sedimented more slowly than IgG and somewhat faster than hemoglobin. In other experiments the material sedimented at a rate intermediate between IgG and BSA markers. Identical patterns were obtained with the ^{125}I-labeled reduced and alkylated specific IgM antibody.

Fig. 5. shows the pattern obtained when 10 μl of ^{125}I-labeled reduced and alkylated IgM antibody was mixed with 2 ml of normal rabbit serum and chromatographed on Sephadex G-200. The IgM subunit was eluted after IgG and before albumin.

Patterns obtained in the ultracentrifuge with markers of graded molecular

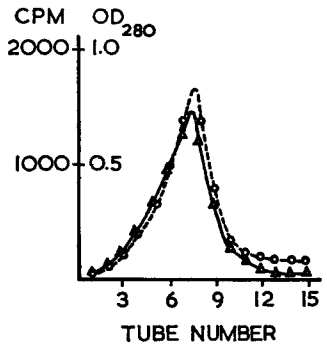


FIG. 6. Ultracentrifugation pattern in a 5–20% sucrose gradient of reduced and alkylated ^{125}I-labeled anti-Forssman IgM mixed with the 5S fragment from pepsin-digested rabbit IgG. Conditions as in Fig. 4. O - - - O, radioactivity of IgM subunits; and ———, OD_{280} of $F(ab')_2$ fragment.

weight showed (Fig. 6) that the subunit sedimented slightly slower than a marker with a molecular weight of about 100,000 (pepsin-digested IgG of known molecular weight and sedimentation coefficient 5S kindly supplied by Dr. S. Utsumi) and considerably faster than rhodamine-labeled BSA with a known molecular weight of 70,000. By interpolation the apparent molecular weight of the subunit is approximately 90,000. A small amount of heavier material with an apparent molecular weight of about 180,000 was noted in most preparations but this was always less than 10% of the total. The apparent molecular weight obtained by density gradient centrifugation could be seriously underestimated if the subunit had an unusual frictional ratio, such as to retard its movement in a centrifugal field. However, any such effect which led to an underestimate of the molecular weight using a centrifugation method should lead to an overestimate using a gel filtration method. Reference to Fig. 5. shows that by gel filtration the apparent size of the subunits was nearer to

IgG (mol wt 150,000) than to albumin. Although this confirms that the value 90,000 for the molecular weight is not grossly erroneous, it can only be regarded as approximate.

Purified samples of the IgM subunit obtained by gel filtration could be further separated into two peaks by gel filtration on Sephadex G-100 with either sodium dodecyl sulfate 0.04 M or guanidine 6 M as eluant (Fig. 7). The two peaks eluted in the positions expected for H and L chains by comparison with IgG light and heavy chains prepared by the method of Utsumi and Karush (15). Although the sizes of the peaks corresponding to H and L chains represent their radioactivities rather than their optical densities, the ratio is the same as the ratio of protein recovery reported by Lamm and Small (4).

Detection of Antibody Activity.—The reduced and alkylated IgM, whether

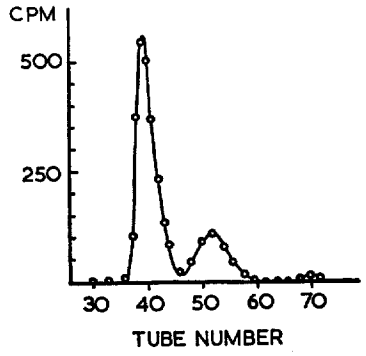
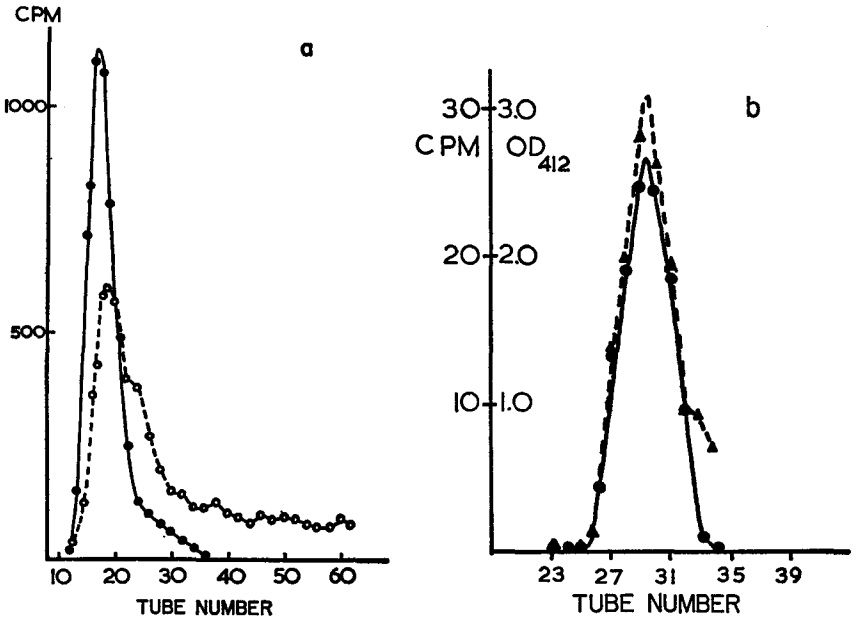


FIG. 7. Separation of H and L chains present in reduced and alkylated ^{125}I-labeled anti-Forssman IgM. Elution from Sephadex G-100 in 0.04 M sodium dodecyl sulfate.

labeled with ^{14}C or ^{125}I, bound only weakly when mixed with excess sheep red cells and centrifuged (the counts specifically attached representing 6–9% in different experiments). We therefore attempted to detect the presence of antibody sites on subunits by allowing aliquots of the reduced and alkylated material to pass through a column contain a 10,000-fold excess of antigen suspended in cellulose. In this way any material with antibody activity would come into contact with antigen repeatedly as it passed down the column. Repeated binding to antigen of antibody subunits, even if their specific binding sites are of low avidity, would result in their retardation compared with the movement of an indifferent marker protein.

On passage of either the ^{14}C-labeled or the ^{125}I-labeled reduced and alkylated IgM antibody through a column of red cell stroma, the elution patterns were as shown in Figs. 8 *a* and 8 *b*. The counts eluted with the indifferent protein marker comprised 50% of those placed on the column (range 46–51% in different experiments). Similar results were obtained with IgM antibody obtained

from individual bleedings from two Sandy Lop and three New Zealand white rabbits. The elution of half the subunits unretarded was not due to the absence of sufficient antigen in the column. This was shown by running the materials which had emerged in the leading peak through a second column of antigen;



FIGS. 8 *a* and 8 *b*. Elution pattern of ^{125}I- or ^{14}C-labeled reduced and alkylated anti-Forssman IgM mixed with an indifferent marker and passed though a column containing sheep erythrocyte stroma. The eluting buffer was VBS with 5% absorbed normal rabbit serum. In each case half the radioactivity of the antibody IgM subunits was retarded.

FIG. 8 *a*. Marker protein was ^{131}I-labeled reduced and alkylated normal IgM, and the column contained stroma equivalent to 12 ml erythrocytes supported on Celite. O – – –O, ^{125}I on antibody IgM subunits; and ●———●, ^{131}I on normal IgM subunits.

FIG. 8 *b*. Marker protein was hemoglobin, and the column contained boiled stroma equivalent to 30 ml erythrocytes supported on cellulose. ▲ – – – ▲, ^{14}C on antibody IgM subunits; and ●———●, OD_{412} of hemoglobin. The retarded labeled subunits were not eluted in this experiment.

the radiolabeled subunits again passed through without retardation, all the radioactivity being recovered with the indifferent marker.

The specificity of the retardation of one-half of the subunits was demonstrated by passing the reduced and alkylated IgM antibody through a similar column prepared with human group B erythrocyte stroma, which does not contain Forssman antigen. In this case 93% of the radioactivity coincided with the marker protein peak (Fig. 9), in contrast to the 50% found with columns of sheep erythrocytes.

The possibility arises that the half of the labeled material which emerged unretarded from antigen columns might be deficient in either H or L chains compared with the starting material, although this is rendered unlikely by the fact that similar findings were obtained using two different methods of labeling which would not be expected to label the two chains differentially to the same extent. The possibility was tested directly in the following way: A complex of IgM antibody and Forssman substance was reduced and alkylated with iodoacetamide-^{14}C as described under method 1, Materials and Methods,

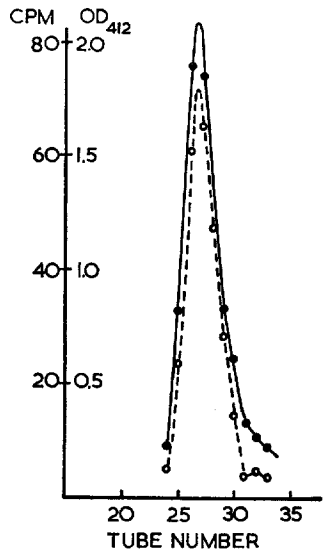


FIG. 9. Elution pattern of ^{14}C-labeled reduced and alkylated anti-Forssman IgM mixed with hemoglobin and passed through a column containing human type B erythrocyte stroma. Conditions as in Fig. 8. The antibody is not retarded. O - - - O, radioactivity of antibody IgM subunits; and ●———●, OD_{412} of hemoglobin.

except that, in order to avoid the presence of carrier rabbit serum proteins, only the subunits released spontaneously were used. This labeled material sedimented as a single peak on centrifugation through a 5–20% sucrose gradient, in a position relative to the markers consistent with a molecular weight of 90,000. It was passed through a very thoroughly washed antigen column (see Materials and Methods), and 47% of the radioactivity was eluted unretarded. Samples of the starting material, of material from the top of the peak, and of material eluted in the descending part of the peak were titrated by the C.F.T. against antisera to rabbit light and μ-chains. The results, set out in Table I, show that both light and heavy chains were present in the unretarded material

and that—within the limits of accuracy of the test—the ratio was the same as in the material before passing through the column.

DISCUSSION

Size of the Rabbit IgM.—Many authors have confirmed the original observation of Deutsch and Morton (16) that IgM dissociated into subunits on reduction and alkylation, and the sedimentation coefficient of these subunits usually been reported as 6–7S. Miller and Metzger (3) studied the structure of the subunits of human IgM from a case of Waldenström's disease, reduced with 0.05 M cysteine and alkylated, and gave evidence for the presence of two H and two L chains. Since the fragments formed on trypsin digestion of

TABLE I

Complement Fixation Technique (Reciprocal of 50% End Point) with Anti-H and Anti-L Chain Sera Given by IgM Subunits before and after Passing Through Antigen Column

	Anti-H 1/80	Anti-L 1/80	cpm
			ml
Starting material	3000	1500	1440/0.1
Unretarded peak	50	90	72/0.1
Titer of unretarded peak expected from dilutions of radioactive material	150	75	
Descending limb of radioactive peak	40	20	
Titer expected from dilution of radioactive material	75	37	
Prepeak eluting buffer	<2	<2	

Doubling dilutions of antigen and fixed dilutions of the antisera was incubated with about three MHD_{50} of C′ at 4°C overnight in microtiter trays. Hemolysis after additions of sensitized erythrocytes and incubation at 37°C was estimated visually.

the subunits were analogous to those formed from IgG, they proposed that the intact IgM molecule may be composed of five linked IgG-like molcules. Suzuki and Deutsch (5, 6) however concluded that the subunits of human IgM paraproteins obtained by mild reduction contain two H and three L chains. The conflict of evidence depends mainly on the accuracy of molecular weight determinations based on measurement of sedimentation and diffusion coefficients.

Our studies with purified rabbit anti-Forssman IgM have indicated that the subunits obtained after reduction with dithiothreitol and alkylation are substantially smaller than IgG, as judged both by their behavior on centrifugation and on filtration through Sephadex G-200. When subjected to ultracentrifugation in a sucrose density gradient the subunits sedimented slower than a marker protein of mol wt 100,000 and faster than a marker protein

of mol wt 70,000 (Fig. 6). The subunits could be dissociated into H and L chains by gel filtration on Sephadex in either sodium dodecyl sulfate or guanidine (Fig. 7). Since the molecular weight of intact rabbit IgM is about 900,000 (4) and the molecular weights of IgM μ-chains and L chains are reported as about 70,000 and 20,000 respectively, our observations suggest that rabbit IgM contains 10 units of molecular weight about 90,000 each made up of one H and one L chain.

The apparent discrepancy between our results and those of others may be due to some unusual property of rabbit anti-Forssman IgM, but is more probably attributable to the fact that our studies were made with very dilute labeled antibody solutions (0.1 mg or less per milliliter) compared with other workers. Other studies were performed with many times more antibody. The discrepancy is probably not due to the method used for reduction and alkylation of IgM, since in another study (to be reported elsewhere) reduction of purified labeled IgM antibody in various concentrations of 2-mercaptoethanol at pH 7.4 also led to the appearance of subunits smaller than IgG. However, when 2-mercaptoethanol was used, reaggregation of the subunits to form 7S and higher complexes occurred to a variable extent, despite alkylation with iodoacetamide and the use of low concentrations of IgM comparable with those used in the present study. Reaggregation of subunits thus appears to take place very readily.

Number of Binding Sites per Molecule.—In a study by equilibrium dialysis of the number of sites on antihapten rabbit IgM molecules, five or six sites were found on the intact molecule and the same number were found on the subunits after reduction and alkylation (1). From structural considerations 10 sites have been postulated (17). Schrohenloher et al. (18) found that in the case of one of three purified rheumatoid factors studied the subunit obtained on reduction contained material which agglutinated IgG-coated erythrocytes. This suggested that the subunits might be bivalent, but the possibility was not excluded that agglutination was brought about simply by their attachment to the coated cells (18). In most investigations IgM subunits, even though they contained four chains, have not been found to have two demonstrable antigen-binding sites.

Under conditions in which subunits consisting of only two chains were obtained from rabbit anti-Forssman IgM, one-half of the subunits could be shown to bind to antigen and one-half could not. Thus it is clear that IgM subunits containing two chains can have an antigen-binding site, just as can two chain subunits of IgG. The problem is to explain why half the subunits failed to bind. One possible explanation is that the avid and nonavid subunits were derived from distinct populations of IgM antibody. This is rendered unlikely by the fact that when subunits were prepared by two quite different methods the proportion which could bind to antigen was the same. In the first method antibody was reduced and alkylated while still complexed to a

purified insoluble antigen. Since antigen was added sufficient to complex with only a part of the total IgM antibody, the complexes would be expected to contain the more avid IgM. In the second method antibody was first dissociated from formolized sheep erythrocytes at alkaline pH, and subsequently reduced and alkylated. Purification by such means would be expected to select for the least avid molecules in the population of antibody. Nevertheless, in both cases precisely half of the subunits could bind to antigen, and the inference is that both the avid and nonavid subunits were originally on the same molecules. A second possible explanation might be that rabbit IgM has 10 H and 15 L chains; that on reduction it breaks into five subunits of two H and three L chains, and that on alkylation two L chains are lost, leaving a subunit containing two H and one L chain as suggested by Suzuki and Deutsch for human IgM (5, 6). At very low concentrations the latter could conceivably break into single H chains and H + L units, of which only the second might bind to antigen. Although our estimates of molecular weight of the subunits did not indicate the presence of such a mixture, they were not accurate enough to exclude it. This explanation is improbable on several grounds, such as the great insolubility of free H chains, and the considerations mentioned on p. 976 but it appears to be ruled out by the demonstration that both H and L chains were present in the subunits which were not retarded on the antigen columns.

If it is accepted that the nonavid subunits are not artefacts due to the methods of preparation and testing, their presence requires accounting for. As a starting point it is clear that in the subunits as isolated (though not in the intact IgM) the H and L chains were not held together by covalent bonds, since they were separable by gel filtration without further treatment. Thus subunits must be the result of preferential association of free chains. Two possible explanations can be put forward to account for obtaining equal numbers of avid and nonavid subunits, both containing one H and one L chain, from the same intact molecules. The first is that in each IgM molecule all the H and all the L chains are identical, but that the separated chains associate in pairs in two equally stable ways, only one of which provides an avid binding site. If the evidence is accepted for the presence of only five binding sites in intact IgM antibodies, the same situation must apply before the interchain S-S bonds are broken. This explanation seems improbable, but is not ruled out. The second is that only half the H chains or half the L chains in an IgM molecule are identical, and that only one combination gives avid subunits. Such an explanation raises serious difficulties if it is taken to imply that there is variation in the amino acid sequence of a given kind of chain made by a single cell. Although Nossal et al. (19) have published evidence that a single cell may make both μ-and-γ heavy chains simultaneously, most evidence from studies of allotypic markers in normal cells and of amino acid sequence in myeloma proteins indicates that at any given time a single cell makes only a homogeneous immunoglobulin

product. It would certainly be simpler to suppose that the amino acid sequences of all the L or all the H chains made by a single cell were identical. Variation between chains could arise if IgM were the complex product of more than one cell, assembled after secretion, or they could conceivably be due to attachment of other groups which have not been identified, or arise through modification of one or more amino acid residues in half the chains (e.g. by conversion of interchain to intrachain disulfide bridges); such modifications might preexist in the original molecule in connection with the bonds holding the chains together or might occur only as a result of breaking these bonds. There is no evidence to support these possibilities, but they cannot be dismissed until more detailed knowledge is available about how the chains are put together in intact IgM molecules.

Relationship between the Number of Binding Sites and Avidity of IgM Antibody.—Hyperimmune rabbit anti-Forssman IgM forms very avid complexes with antigen (7, 8), even though the subunits composed of one H and one L chain bind at best weakly. However our studies (to be reported) with IgM antibody reduced with 2-mercaptoethanol and alkylated, but under conditions in which reaggregation of the subunits occurs, show that the presence of two or more binding sites on the aggregates greatly increases their ability to bind specifically to antigen on erythrocyte surfaces. This is in accord with the finding of Greenbury et al. (20) who demonstrated for rabbit IgG antibody against human group A_1 erythrocytes that univalent F(ab) or F(ab)′ fragments had a much smaller association constant in their reaction with erythrocytes than had the original IgG or the bivalent $F(ab)^2$.

The subunits of rabbit IgM anti-*p*-azobenzene-arsonate are reported to bind hapten with the same avidity as the intact IgM, with an association constant not greatly dissimilar from that of an IgG antibody against the same hapten (1). However, in one instance when the size of the binding sites of IgM antibody against a polysaccharide antigen was examined, these were found to be small compared with the sites on corresponding IgG antibodies. Thus the binding of human anti-A IgM, unlike IgG, was inhibited even by a monosaccharide hapten (21). Our observation of weak binding to Forssman antigen by IgM subunits is also consistent with the presence of a small binding site. Even when IgM antibodies have only weak bindings sites they may nevertheless be expected to bind firmly to materials which have areas of repeated antigenic structures at their surface sufficiently close together to permit multipoint attachment.

SUMMARY

Rabbit IgM anti-Forssman antibody was highly purified and the subunits obtained on reduction and alkylation were labeled radioactively and isolated by two different and unrelated methods. In both cases the subunits were found

to have a molecular weight of about 90,000, based on their behavior on density gradient centrifugation and gel filtration, and evidence is given that they contained one light and one heavy chain. The subunits bound only weakly to sheep erythrocyte stroma, and only half could be shown to possess antigen specific sites. The data are consistent with the concept that each anti-Forssman IgM molecule has five effective binding sites, but it is uncertain whether the ineffectiveness of the remaining five H-L chain pairs is inherent in the structure of the IgM molecule or an artefact due to the isolation procedure. Intact IgM anti-Forssman antibody binds very firmly to structures containing multiple repeating antigen sites, and it appears that this is due to the presence of multiple binding sites on the molecule.

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