Conformation of Immunoglobulin M. 1. Characterization of Anti-ε-1-Dimethylamino-5-naphthalenesulfonyl-L-lysine Immunoglobulin M Antibodies from Horse, Pig, and Shark†

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ABSTRACT: IgM antibodies specific for the fluorophore ε-1-dimethylamino-5-naphthalenesulfonyl-L-lysine (DNS-lysine) were elicited in the horse and nurse shark by immunization with a DNS-lysine streptococcal conjugate; the antibodies were purified by specific adsorption with an immunoadsorbent followed by gel filtration to select the IgM class (molecular weight 900 000). About 90% of the equine anti-DNS was IgM. DNS-Lysine, when bound in the combining sites of a population of these anti-DNS IgM antibodies from horse and nurse shark, as well as from pig, exhibited a marked fluorescence enhancement and shift of the emission spectrum to shorter wavelengths compared with emission in aqueous solution; these results indicate that the environments of the anti-DNS combining sites of this population were relatively hydrophobic. Approximately one-third of the ten possible combining sites in each of these anti-DNS IgM species bound DNS-lysine in this manner with an average intrinsic association constant (K_0) of greater than 10^6 M^{-1}. Small differences were noted in binding behavior among the three species of antibodies. The enzymatic susceptibility of equine IgM was similar to that of human IgM. $(Fab')_2\mu$, $Fab'\mu$, and $Fab\mu$ fragments were prepared following digestion with pepsin. These fragments could be clearly differentiated on the basis of molecular size. They bound DNS-lysine with the same affinity as intact IgM and the DNS-lysine–fragment complexes exhibited the same spectral properties as the parent IgM. It was concluded that the anti-DNS IgM antibodies from all three species, as well as the enzymatic fragments, were suitable for nanosecond depolarization studies which are reported in the accompanying paper Holowka, D. A., and Cathou, R. E. (1976), *Biochemistry,* the following paper in this issue.

Since the initial observations of Heidelberger and Pedersen (1937) of a macroglobulin antibody against *Pneumococcus* in the horse, immunoglobulin M (IgM)[1] has been recognized as a major class of antibodies in humans and other mammals, as well as in vertebrates as phylogenetically distant as the elasmobranchs (sharks). In serum it is generally found as a pentamer of five disulfide-linked subunits, each composed of two light (L) and two heavy (μ) chains which are held together by disulfide bridges and noncovalent interactions (Metzger, 1970). A third unrelated chain, known as the J chain, is also present in IgM and is probably involved in its assembly from the subunits (Koshland, 1975). The overall molecular weight of the pentamer is about 900 000 (Dorrington and Tanford, 1970). Functionally, the IgM pentamer has ten potential antigen binding sites, one on each $Fab\mu$ (Kim and Karush, 1973; Riesen et al., 1975), and is capable of binding C1q in the $(Fc)_5\mu$ region to initiate the classical complement pathway (Sledge and Bing, 1973; Müller-Eberhard, 1974). The 8S subunit is thought to act as the major cell surface receptor for antigens on small B lymphocytes (Warner, 1974).

The recent elucidation of the entire amino acid sequence of two human μ chains (Putman et al., 1973; Watanabe et al., 1973) has made possible a more complete understanding of the structure and conformation of IgM. In particular, the discovery of a fifth domain, $C\mu2$, which separates the $Fab\mu$ from the $(Fc)_5\mu$ core has made the question of segmental flexibility in this antibody an especially intriguing one. Since a single IgM antibody is capable of binding the C1q to initiate the complement cascade (Borsos and Rapp, 1965), a change in the noncovalent interaction of the $C\mu2$ domains within an IgM subunit might mediate a signal from the antigen binding sites on the $Fab\mu$ segments to the $(Fc)_5\mu$ region where C1q is bound (Sledge and Bing, 1973; Metzger, 1974). Some recent studies have also indicated that IgM molecules from different species vary significantly with respect to certain structural features: porcine IgM has been found to be much more resistant to proteolysis and reductive cleavage than human IgM (Beale, 1974b), and steady-state fluorescence polarization parameters have been found to exhibit species-dependent variation (Zagyansky, 1975).

In order to study the question of segmental flexibility of IgM, we have taken advantage of the observation that certain species produce a strong IgM response to bacterial vaccines (Heidelberger and Pedersen, 1937; Clem and Leslie, 1971; Kim and Karush, 1973) and have elicited IgM antibodies in the horse and nurse shark to a fluorescent *hapten,* DNS-lysine, which was covalently coupled to heat-killed *Streptococcus* vaccine. In this paper we describe the purification and characterization of these anti-DNS antibodies and their enzymatic fragments, and of anti-DNS IgM from the pig. In the subse-

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[1] Abbreviations used: DNS-lysine, ε-1-dimethylamino-5-naphthalenesulfonyl-L-lysine; anti-DNS, antibodies elicited to the DNS-lysine hapten which show measurable binding of the DNS determinant; DNS–HSA, DNS covalently conjugated to human serum albumin; dansic acid, dimethylaminonaphthalenesulfonic acid; KLH, keyhole limpet hemocyanin; Tris, tris(hydroxymethyl)aminomethane; 2-ME, 2-mercaptoethanol; C1q, first component of complement; IgG, immunoglobulin G; IgM, immunoglobulin M; L, light chain; μ, heavy chain; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethylchloromethyl ketone; DNP, dinitrophenyl.

quent paper (Holowka and Cathou, 1976) we report studies of these antibodies which utilize the technique of nanosecond fluorescence depolarization in an effort to ascertain the extent and location of segmental flexibility in IgM.

Materials and Methods

Preparation of Antigen and Immunization. The antigen was prepared by a method suggested by L. W. Clem (private communication). Group C *Streptococci,* strain C74, were cultivated, heat-killed, and pepsin treated (Krause, 1970). One hundred milliliters of this *Streptococcus* vaccine was centrifuged, the pellet was resuspended in 10 ml of H_2O, at pH 11, and 25 ml of a freshly prepared, saturated aqueous solution of cyanogen bromide was added. After 5 min at 4 °C with constant stirring, the cells were quickly pelleted, washed once with 0.1 M $NaHCO_3$, resuspended in 40 ml of 5 mM DNS-lysine (Sigma Chem. Co.), 0.1 M $NaHCO_3$, pH 8.5, and stirred at 4 °C for 20 h. After about six washes with 50 ml of Tris–saline 7.5 buffer (0.02 M Tris, 0.15 M NaCl, pH 7.5), the absorbance of the wash solution at 330 nm was less than 0.05, and the cells were resuspended in 100 ml of the Tris–saline 7.5 buffer with 0.02% formalin. The degree of conjugation of DNS-lysine was 0.05 to 0.08 nmol per 100 ml of vaccine (ca. 4 mg/ml *Streptococci*).

A horse was intravenously immunized with this vaccine (DNS-lysine-strep) with an initial course consisting of three injections per week (15–50 mg *Streptococci* per injection) for 3 weeks, followed by a second series 3 months later of four injections over the course of 3 weeks. The horse was then bled the day after the last injection. Serum (4 l.) was squeezed from blood clotted at room temperature, and anti-DNS antibodies in the serum were initially identified by specific precipitation with DNS–conjugated human serum albumin (DNS–HSA).

Nurse sharks (20–40 lb each) were immunized with the same vaccine by Dr. L. W. Clem of the University of Florida; they received an initial intravenous injection of about 40 mg of DNS-lysine-strep, followed after 2 months by boosts of 60 and 100 mg on two successive days. Antiserum (450 ml) from a bleed 2 months after the last injection could precipitate DNS–HSA, and this precipitation was specifically inhibited by DNS-lysine.

Purification of IgM Anti-DNS. Anti-DNS antibodies were purified from the immune sera by selective adsorption of 50–100 ml of serum with DNS–HSA–bromoacetylcellulose or naphthalene–HSA–bromoacetylcellulose (Robbins et al., 1967) followed by exhaustive batchwise washing of the immunoadsorbent with Tris–saline 8 buffer (0.02 M Tris, 0.15 M NaCl, pH 8.0), and finally elution by a solution of either 1.0 M acetic acid, pH 2.5–3.0 for 1 h at room temperature (acid-eluted), or 0.15 M dansic acid (Pierce Chem. Co.) for 15 h at 4 °C (hapten-eluted). Eluates (20–30 ml) were dialyzed vs. two to three changes of 8 l. of Tris–saline 8 buffer. The solution of anti-DNS antibodies was concentrated to 10–15 ml by ultrafiltration at 4 °C with a Diaflo PM-30 membrane (Amicon, Lexington, Mass.), and then chromatographed on a column of Sephadex G-200 (2.5 × 95 cm) or Sepharose 4B (2.5 × 90 cm) (Pharmacia). All chromatography was performed in 0.02 M Tris, 0.50 M NaCl buffer, pH 8.0, at room temperature. Pooled fractions were concentrated as above and filtered with a MF-Millipore 0.22-μm filter (Millipore Corp.) before further analysis. Analytical ultracentrifugation showed the absence of aggregated material.

Partially purified porcine anti-DNS IgM was generously supplied by Dr. F. Franěk of the Institute for Organic Chemistry and Biochemistry, Prague, Czechoslovakia. The antibodies were elicited by immunization with DNS–KLH and were purified by elution from a DNS immunoadsorbent with 0.2 M acetic acid followed by immediate neutralization (F. Franěk, personal communication). They were further purified in this laboratory by chromatography on either Sephadex G-200 or Sepharose 4B (2.5 × 75 cm) as described above.

Preparation of Equine IgM Fragments. Acid eluted equine anti-DNS antibodies were digested with pepsin according to the procedure used for human IgM (Kishimoto et al., 1968); the digest was then chromatographed on Sephadex G-200. After an initial survey with sodium dodecyl sulfate gel electrophoresis, the desired fractions were pooled and concentrated for further analysis. In some cases, the fraction identified as the Fabμ fragment was further digested under nonreducing conditions with papain (Onoue et al., 1968) for 10 h and then chromatographed immediately on Bio-Gel P-150 (1.5 × 95 cm; Bio-Rad). Digestions of whole equine IgM with papain under nonreducing conditions (Onoue et al., 1968) and Tos-PheCH$_2$Cl-trypsin at 60 °C for 29 min (Plaut and Tomasi, 1970; Zikán and Bennett, 1973) were performed under the same conditions as prescribed for the production of the (Fc)$_5\mu$ fragment from human IgM.

Reduction and Alkylation of Equine (Fab')$_{2\mu}$. 2-Mercaptoethanol (2-ME) was added to a solution of (Fab')$_{2\mu}$ (0.2%) to give a final concentration of 0.2 M 2-ME in a solution buffered to pH 8.1 with 0.2 M Tris-HCl and which had been first deoxygenated by streaming nitrogen over its surface for 20 min. Reduction was allowed to continue for 1 h at room temperature, followed by alkylation at 4 °C for 15 min with 0.22 M iodoacetamide. The solution was then dialyzed at 4 °C vs. Tris–saline 8 buffer. A small amount of protein which appeared to be aggregated (by gel electrophoretic analysis) was effectively removed by centrifugation of the sample at 100 000g for 2 h.

Analytical Techniques. Immunoelectrophoresis was performed as described by Feinstein (1968) using 0.02 M sodium barbital, 0.025 M sodium acetate, buffer pH 8.6. Rabbit anti-horse IgG (heavy and light chain specific) and anti-whole horse serum antisera were purchased from Cappel Laboratories, Downington, Pa. Goat anti-human IgM antiserum was purchased from Meloy Laboratories, Springfield, Va.; we found that this product was μ-chain specific. The rabbit anti-porcine serum was a commercial antiserum given to us by Dr. Franěk. Human IgM was obtained from the American National Red Cross (Bethesda, Maryland). Double immunodiffusion (Ouchterlony, 1949) was performed using 1% agar plates.

Sodium dodecyl sulfate gel electrophoresis with 5% polyacrylamide gels in a Shandon apparatus employed the method of Weber and Osborn (1969), except that 2-ME was omitted. The semilog calibration plot of molecular weight vs. mobility was linear as long as the standard proteins were all unreduced. Electrophoresis under nondissociating conditions employed the uniform pH-discontinuous voltage gradient method of Maurer and Allen (1972), with 5% acrylamide gels and an electrode solution of 0.05 M Tris, 0.375 M glycine buffer, pH 8.5. Gel and sample solutions were 0.375 and 0.0375 M in Tris, respectively. Protein mobilities were calculated as the ratio of the distance of migration of the stained protein to that of the tracking dye.

Sedimentation velocity experiments were performed on a Spinco Model E analytical ultracentrifuge, employing in most cases the AN-E rotor and schlieren optics. Protein concentrations ranged from 0.25 to 3.0 mg/ml and all samples were dialyzed vs. Tris–saline 8 buffer.

Fluorescence Spectra and Titrations. Corrected fluorescence emission spectra and titrations were recorded on an Aminco SPF 1000 spectrofluorometer (American Instrument Co.) in a mode which provided continuous compensation for small changes in the lamp intensity (Kremen and Landa, 1974). Solutions (1.5 ml) were routinely excited at 330 nm, and A_{330nm} was at all times less than 0.10. Relative fluorescence enhancement of bound DNS-lysine was determined from the first few increments of hapten added to antibody at concentrations at which essentially all of the added hapten was bound.

The binding isotherm of DNS-lysine to anti-DNS antibody, as measured by fluorescence enhancement of the bound DNS-lysine at 480 or 500 nm, was analyzed by the method of Scatchard (1949). In this analysis, the fluorescence of the antibody–hapten solution was assumed to be completely described by the equation:

$$F = q_1(\mathrm{AH}) + q_2 c \quad (1)$$

where F is the total observed fluorescence, q_1 is the fluorescence per unit of bound hapten, q_2 is the fluorescence per unit of free hapten, and (AH) and c are the concentrations of bound and free hapten, respectively (Parker et al., 1967b). q_1 was estimated from the initial additions of DNS-lysine to the antibody solution, and q_2 was obtained from the final additions of DNS-lysine. Combining eq 1 with eq 2

$$H_0 = (\mathrm{AH}) + c \quad (2)$$

where H_0 is the total hapten concentration, one obtains the following relationship:

$$(\mathrm{AH}) = \frac{q_2 H_0 - F}{q_2 - q_1} = rA_0 \quad (3)$$

where A_0 is the total antibody concentration and r is the number of moles of hapten bound per mole of antibody.

Thus, r may be calculated for each point in the titration, and calculation of c from eq 2 permits the plotting of r/c vs. r. K_0, the average intrinsic binding constant at half saturation of sites, and n, the maximum number of sites, are then obtained graphically (Karush and Karush, 1971). The concentration of IgM used in the titrations was usually about 10^{-6} M, assuming a molecular weight of 900 000, and an extinction coefficient of $\epsilon_{1\,mg/ml} = 1.35$ (Kim and Karush, 1973). A total volume of approximately 50 μl of an aqueous solution of DNS-lysine was added in varying increments to an initial volume of 1.5 ml; no correction was made for dilution of the antibody solution. For the emission spectra of bound DNS-lysine, antibody solutions were titrated and then dialyzed overnight vs. 4 l. of Tris–saline 8 buffer to remove any unbound DNS-lysine.

Results

Purification of Anti-DNS IgM Antibodies. When equine anti-DNS antibodies were purified as described above, a yield of about 1.2 mg/ml of serum was obtained. No significant differences were observed in the fluorescence titrations of antibodies prepared with either the naphthalene or DNS immunoadsorbents, although the adsorbing capacity of the former immunoadsorbent was lower. Neither were there any significant differences in the spectral properties and titrations of the hapten-eluted vs. acid-eluted antibodies. As shown in Figure 1, on Sephadex G-200 chromatography of the anti-DNS antibody, about 90% of the protein emerged in the void volume, and the remaining 10% in a second band. Sedimentation velocity analysis of the void volume fraction gave a single

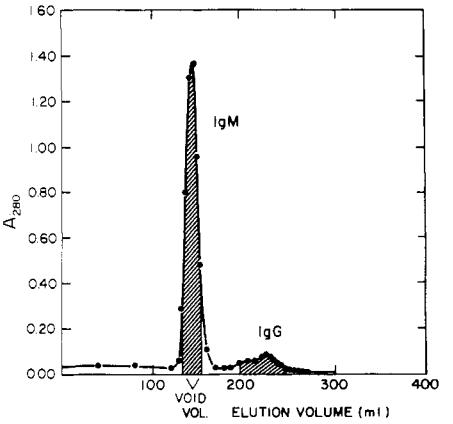

FIGURE 1: Sephadex G-200 fractionation of immunoadsorbent-purified equine anti-DNS antibody. Hapten-eluted antibody (22 mg) was chromatographed in Tris-saline 8 buffer; diagonal lines indicate fractions which were pooled, concentrated, and filtered (0.22 μm, Millipore) before immunochemical identification.

symmetrical boundary with an $s_{20,w}{}^0$ value of 17.5 S. Sodium dodecyl sulfate–acrylamide gel analysis of this material showed less than 4% contamination by IgG. Double immunodiffusion analysis of the second chromatography band with anti-horse IgG antiserum indicated that it was chiefly IgG, but no further characterization was made. Sepharose 4B chromatography yielded similar results and was actually the preferred method because it consistently gave better separation of IgM from IgG and also assured separation of IgM from aggregates or suspended immunoadsorbent particles.

The concentration of anti-DNS antibody in the nurse shark serum was considerably lower than that in the horse serum; a maximum yield of 0.15 mg/ml of serum was obtained. Because acid elution of this antibody from the DNS immunoadsorbent caused considerable protein aggregation, only hapten elution was employed. Sephadex G-200 chromatography separated the purified shark anti-DNS antibody into two components: a major component which emerged in the void volume, and accounted for at least 75% of the eluted protein, and a second component which appeared as a shoulder on the void volume band and was pooled separately. The latter material bound DNS-lysine and probably represents the 7S IgM species (Clem et al., 1967). The first component was pooled and concentrated and found to bind DNS-lysine with an affinity similar to that observed for the other anti-DNS IgM species (see below). It exhibited a single schlieren boundary with a $s_{20,w}$ value of 18.3 S at 0.49 mg/ml, and will be referred to as nurse shark IgM. From sodium dodecyl sulfate–gel electrophoretic analysis, it appeared to be contaminated less than 8% by the low molecular weight antibody. Immunoadsorbent-purified anti-DNS from a second nurse shark was chromatographed on Sepharose 4B, which resulted in a complete separation of 19S IgM from 7S IgM. Although the fluorescence properties of DNS-lysine bound to this IgM were slightly different (Holowka, 1975), the nanosecond depolarization analysis was essentially the same (Holowka and Cathou, 1976).

On Sepharose 4B chromatography of the porcine anti-DNS preparation, 90% of the material emerged as a single symmetrical band after the void volume and exhibited a $s_{20,w}$ value of 17.2 S at a protein concentration of 2.1 mg/ml. Sodium dodecyl sulfate–polyacrylamide gel analysis of 100 μg of this IgM showed no trace of lower molecular weight contaminants. The remaining 10% of protein applied to the Sepharose 4B column emerged as a second separate peak and was not analyzed.

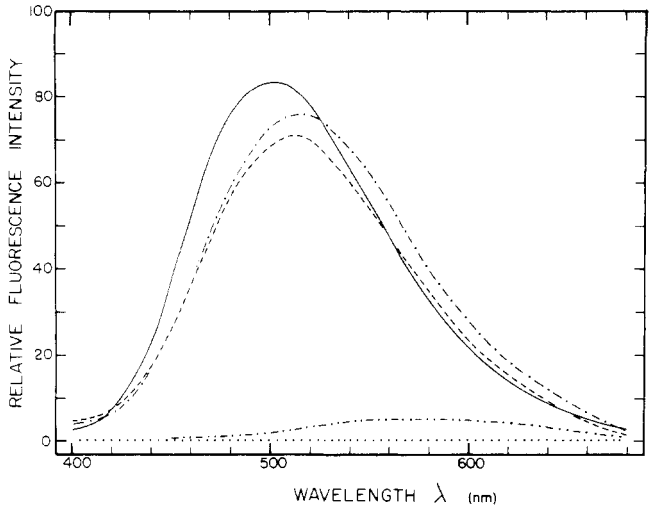

FIGURE 2: Corrected emission spectra of the DNS-lysine–anti-DNS IgM complexes. The concentration of DNS-lysine in the anti-DNS complexes is 2.5×10^{-6} M, the same as that of DNS-lysine in Tris-saline 8 buffer. Excitation was at 330 nm (5-nm spectral band width). Emission band width was 10 nm. (—) Equine anti-DNS IgM; (- - -) porcine anti-DNS IgM; (- · -) nurse shark IgM; (- · · -) DNS-lysine in buffer; (· · ·) buffer baseline.

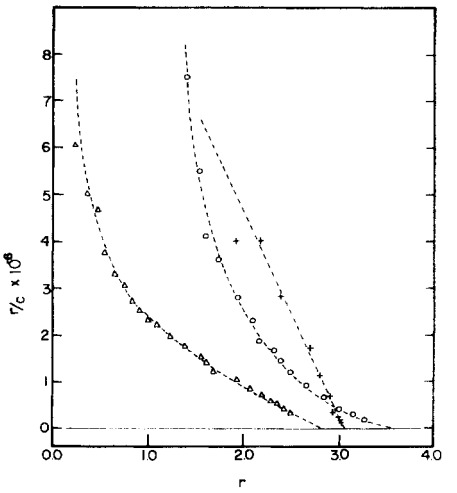

FIGURE 3: Scatchard plots of the DNS-lysine–anti-DNS IgM fluorescence enhancement binding isotherms. r is the number of moles of DNS-lysine bound per mole of anti-DNS IgM, and c is the molar concentration of unbound DNS-lysine present. (△) Porcine anti-DNS IgM; (○) equine anti-DNS IgM; (+) nurse shark anti-DNS IgM.

TABLE I: Spectral and Binding Properties of DNS-Lysine–Anti-DNS IgM Complexes.

	λ_{max} Emission (nm)	Enhancement vs. Aqueous Soln at 500 nm	K_0 (M^{-1})	n
DNS-Lysine bound to:				
Equine IgM (hapten eluted)	502	40×	2.0×10^6	3.5
Equine IgM (acid eluted)	502	40×	1.8×10^6	3.7
Porcine IgM	511	35×	1.1×10^6	2.8
Nurse shark IgM	515	37×	4.5×10^6	3.0
DNS-Lysine in 99% dioxane	503	26×		

From immunoelectrophoretic analysis, it was found that the goat anti-human IgM antiserum gave a single precipitin line with both porcine IgM and equine IgM (acid- and hapten-eluted), but not with nurse shark IgM. Moreover, the reaction seemed to be Fcμ specific since porcine Fabμ and equine $(Fab')_2\mu$ did not cross-react, while the porcine $(Fc)_5\mu$ did. Likewise, antisera against whole equine, porcine, and nurse shark sera gave only a single line with the respective purified IgM preparation in double immunodiffusion analysis. Analysis of the J chain content of the equine IgM showed about 0.7 mol/mol IgM, which is in the range to be expected in the absence of full precautions to inhibit esterase activity (Brown and Koshland, unpublished results and personal communication).

DNS-Lysine–Anti-DNS IgM Complexes. The binding of DNS-lysine by anti-DNS IgM from all three species, horse, pig, and shark, was accompanied by a marked fluorescence enhancement and shift of the emission maximum to shorter wavelengths, relative to that seen in aqueous solution, in a similar fashion to the behavior previously observed on binding to rabbit IgG anti-DNS (Parker et al., 1967a). As can be seen in Figure 2 and Table I, the magnitude of the blue shift of the emission maximum was somewhat different for each of the three IgM species, with the largest shift occurring for DNS-lysine bound to equine anti-DNS IgM. Note also that the relative enhancement of fluorescence was not directly related to the wavelength of maximum emission and, in particular, that the enhancement observed for DNS-lysine in dioxane was only 65–75% of that seen when DNS-lysine was bound to the anti-DNS IgM. Such behavior is probably due to factors other than the dielectric constant of the fluorophore's environment (Greene, 1975; Brand and Gohlke, 1972).

The extent of binding of DNS-lysine to each of the three species of IgM was determined by measuring the fluorescence enhancement at 480 or 500 nm of small aliquots of hapten added sequentially to an IgM solution until only background changes in fluorescence were observed. The titration data were then analyzed by means of Scatchard plots, as described in Methods (see Figure 3). The purified equine and porcine antibodies contained small amounts of residual bound hapten which appeared, from fluorescence behavior, to be dansic acid. If one assumes that dansic acid and DNS-lysine exhibit the same value of q_1, then about 5 to 20% of the measurable binding sites were occupied by dansic acid. In the case of nurse shark anti-DNS IgM, this intrinsically bound hapten occupied 40–60% of the total measureable binding sites. In all cases, the Scatchard analyses included these prefilled sites. The average intrinsic affinity constant, K_0, and number of total measurable binding sites, n, for each of the anti-DNS IgM species are shown in Table I. For all three IgM species, the value of K_0 is high relative to values reported for anti-hapten IgM antibodies with valencies near 10 (Kim and Karush, 1973; Congy et al., 1974; Voss and Sigel, 1972). It should be noted that only about a third of the ten possible combining sites could be titrated under these conditions so that only the highest affinity anti-DNS antibodies are contributing to fluorescence enhancement of DNS-lysine. However, at least 90% of the purified equine IgM (acid eluted) could be readsorbed onto the immunoadsorbent so that almost all antibodies were capable of binding at least one hapten. These titratable sites may correspond to the high affinity IgM population observed after multiple immunizations in rats (Oriol and Rousset, 1974), rabbits (Onoue et al., 1968), and nurse sharks (Voss and Sigel, 1972).

Enzymatic Fragments of Equine IgM. $(Fab')_2\mu$ and Fabμ fragments were obtained when acid-eluted equine anti-DNS

antibody, 90% IgM (eluted from immunoadsorbent but not chromatographed), was digested with pepsin and then subjected to Sephadex G-200 chromatography. $(Fab')_2\mu$ was eluted in the first peak which appeared after emergence of the void volume; the fragment moved as a single band on sodium dodecyl sulfate gel electrophoresis and had an apparent molecular weight of 105 000–120 000 (Metzger, 1970).

Fabμ was eluted in the third peak and consisted of a major component with an apparent molecular weight of 56 000 and minor component of 44 000; the latter accounted for less than 12% of the total stained protein. Although the major component exhibited the same mobility as that of the Fabμ fragment prepared from human IgM by hot trypsin digestion (Plaut and Tomasi, 1970), the apparent molecular weight was larger than that expected (50 000); it was therefore subjected to papain digestion under nonreducing conditions (Onoue et al., 1968). Chromatography of the digested material on Bio-Gel P-150 resulted in a major product which was eluted at the same volume as rabbit Fabμ prepared by papain cleavage of $(Fab')_2\mu$ and which was pooled for further characterization. Sodium dodecyl sulfate gel analysis showed that the equine papain Fabμ fragment had been cleaved into two polypeptide chain products, one with an apparent molecular weight of 33 000, and the other 17 000, each present in approximately equal amounts. This fragment, as well as the $(Fab')_2\mu$ and the pepsin Fabμ, bound DNS-lysine with the same affinity as intact equine IgM and the DNS-lysine complex exhibited the same spectral properties. In double immunodiffusion, all fragments gave a precipitin line with anti-horse IgG (L-chain specificity) but not with anti-human IgM (Fcμ cross-reactive; see above). Both the papain Fabμ and the pepsin Fabμ exhibited the same elution volume on Sephadex G-200 (see below). They also yielded identical rotational correlation times by nanosecond depolarization (Holowka and Cathou, 1976). The only observable effect of papain thus was nicking of the polypeptide chains.

In order to determine whether the equine $(Fab')_2\mu$ fragment remained associated following reduction of the inter-heavy-chain disulfide bond, the fragment was subjected to reduction with 0.2 M 2-ME and alkylated with iodoacetamide. Sodium dodecyl sulfate–acrylamide gel electrophoresis showed the presence of Fab'μ, some free light and heavy chains, and the absence of any unreduced $(Fab')_2\mu$. Comparative chromatography of Fab'μ, $(Fab')_2\mu$, and Fabμ (papain) on Sephadex G-200 is illustrated in Figure 4. In all cases the elution pattern of absorbance at 280 nm corresponded to that of the fluorescence of bound DNS-lysine. It is evident that Fab'μ eluted in a position between $(Fab')_2\mu$ and Fabμ (papain), indicating that interaction between the Cμ2 domains was not sufficient to keep the Fab'μ moieties associated. Similar results have been obtained with human IgM fragments (Shimizu et al., 1974). From chromatography of a pepsin digest on the same column, it was found that Fabμ (pepsin) eluted at the same volume as Fabμ (papain) (not shown). When equine fragments were subjected to electrophoresis under nondissociating conditions (see Methods), the results were compatible with those described above, with $(Fab')_2\mu$, Fab'μ, and Fabμ (pepsin) showing average mobilities of 0.16, 0.29, and 0.31, respectively.

Initial attempts to prepare an $(Fc)_5\mu$ fragment from *acid-eluted* equine IgM were unsuccessful. Papain under nonreducing conditions at 37 °C (Onoue et al., 1968), and Tos-PheCH$_2$Cl-trypsin at 60 °C (Zikán and Bennett, 1973) yielded only smaller fragments and peptides. However, digestion of *hapten-eluted* equine IgM with Tos-PheCH$_2$Cl-trypsin at 60 °C for 20 min resulted in the production of fragments with

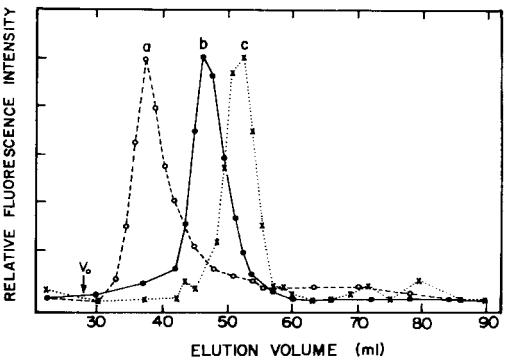

FIGURE 4: Comparative G-200 chromatography of acid-eluted equine IgM enzymatic fragments. DNS-lysine bound to the titrated anti-DNS IgM fragments was monitored by exciting at 330 nm and observing the fluorescence at 480 nm. (a) $(Fab')_2\mu$ (1.6 mg); (b) Fab'μ (0.32 mg); (c) Fabμ (papain) (1.5 mg). V_0 is the void volume of the column; the relative fluorescence has been normalized with respect to that of Fab'μ.

mobilities on sodium dodecyl sulfate–acrylamide gel electrophoresis which corresponded to those of $(Fc)_5\mu$ and Fabμ from human IgM. Thus, the 1 M acetic acid treatment during purification appears to make the $(Fc)_5\mu$ region more susceptible to tryptic hydrolysis.

Discussion

Production of Anti-Hapten IgM. DNS-lysine covalently coupled to heat-killed, pepsin-treated *Streptococci* proved to be an effective immunogen for hapten-specific antibody in the horse. After a third boost and subsequent bleed, the total quantity of anti-DNS antibody from 8 l. of serum from this horse was about 10 g, 90% of which was IgM. Since anti-hapten antibodies in the IgM class are usually difficult to elicit in significant quantities, this method provides a means to obtain the quantities necessary for physicochemical and structural studies. The magnitude of the IgM response may, however, be dependent on the particular hapten employed. We have also attempted to elicit equine anti-pyrene antibodies by the same method; only traces of anti-pyrene IgM were found (Holowka, 1975). However, because of the large volumes of serum that can be withdrawn from a horse, one can still obtain significant quantities of antibody. The concentration of anti-DNS antibody from nurse shark was found to be much lower than that found in the horse, despite the fact that anti-*Streptococcus* antibody can be elicited in the nurse shark in concentrations as high as 10 mg/ml (Clem and Leslie, 1971). It is, however, similar to the quantities of anti-dinitrophenyl antibodies elicited by DNP-KLH (Voss et al., 1969).

Combining Sites of the Anti-DNS IgM Antibodies. Of the IgM preparations studied, the binding of DNS-lysine by equine anti-DNS IgM results in an emission maximum blue shift which is most similar to that observed in the rabbit IgG anti-DNS–DNS-lysine complex (Parker et al., 1967a; Werner et al., 1972). It has been recently shown that aminonaphthalenesulfonamides such as DNS-lysine show a fairly direct correlation between emission maxima and the polarity of their environment (Greene, 1975), and thus relatively apolar anti-DNS combining sites are implicated in the observed DNS-lysine binding. Furthermore, the equine antibody combining site shows a number of other indications of a hydrophobic environment: the corrected excitation maximum of bound DNS-lysine is 335 nm compared with 326 nm for DNS-lysine in aqueous solution, and the fluorescence lifetime is long (22 ns), while the decay is essentially single exponential (Holowka and Cathou, 1976). In agreement with this latter observation,

the emission spectrum is only slightly dependent on excitation wavelength (Holowka, 1975). This uniformity of the emitting species in equine anti-DNS IgM is significant in light of the excitation wavelength dependence of the rotational correlation time of its Fabμ fragment (Holowka and Cathou, 1976) since it argues against combining site heterogeneity as the explanation for the latter observation.

The porcine IgM anti-DNS combining sites, however, are clearly different from the equine. Besides the less marked blue shift of the emission spectrum of bound DNS-lysine, the emission maximum is quite highly dependent on the excitation wavelength, shifting from 508 nm with λ_{ex} 320 to 520 nm with λ_{ex} 385 nm (Holowka, 1975). Since the excitation spectrum is also dependent on the wavelength of emission (unpublished observation), it is quite probable that there are a number of different types of combining sites from which the bound DNS-lysine is emitting (Schlessinger et al., 1974). The presence of more than one fluorescence lifetime (Holowka and Cathou, 1976) is also indicative of heterogeneity. It is not known why the combining sites of porcine anti-danysl IgM should be more heterogeneous with respect to binding DNS-lysine and appear to be less hydrophobic than the equine sites, but the difference in carriers used in immunization (*Streptococci* vs. KLH) may be a factor (Brenneman and Singer, 1970).

In the case of the shark anti-DNS IgM, it is difficult to determine how much of the dansic acid is displaced by DNS-lysine upon addition of the latter and, hence, a direct comparison of the properties of the combining site with those of the other two species is not warranted.

Structural Contrasts between Equine and Porcine IgM. The finding that IgM from the pig and the horse (acid *or* hapten eluted) cross-reacted with an anti-human IgM antiserum is consistent with the high cross-reactivity among nonprimate IgM species previously observed with a similar antiserum (Mehta et al., 1972). This cross-reactivity is in contrast to the significant differences in enzymatic susceptibility observed among the $(Fc)_5\mu$ moieties of porcine, human, and equine IgM. For example, although both human and hapten-eluted IgM are cleaved by trypsin at 60 °C to yield an $(Fc)_5\mu$ fragment, porcine IgM is resistant to such treatment and yields $(Fc)_5\mu$ only with pepsin (Beale, 1974b); the latter enzyme digests $(Fc)_5\mu$ of human and equine IgM. Also, pepsin digestion of porcine IgM yields Fabμ rather than $(Fab')_2\mu$ (Beale, 1974a,b; Zikán and Miler, 1974). Stronger noncovalent interactions also exist in the Fc region of porcine IgM (Beale, 1974a,b; Hester et al., 1975). Nevertheless, the flexibility of the molecule as determined by nanosecond fluorescence depolarization is similar to that of native equine IgM (Holowka and Cathou, 1976). These apparent discrepancies might be explained by the conservation of most of the primary sequence in the constant domains from these different mammalian species, but with limited amino acid substitutions, such as in charged residues at the polypeptide surface, that could markedly affect interdomain noncovalent interactions. The dissociation of succinylated concanavalin A to dimers is an example of this effect (Gunther et al., 1973; Reeke et al., 1975).

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Conformation of Immunoglobulin M. 2. Nanosecond Fluorescence Depolarization Analysis of Segmental Flexibility in Anti-ϵ-1-Dimethylamino-5-naphthalenesulfonyl-L-lysine Anti-Immunoglobulin from Horse, Pig, and Shark†

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ABSTRACT: The rotational motions of immunoglobulin M (IgM) were investigated by the nanosecond fluorescence depolarization technique. The fluorophore ϵ-1-dimethylamino-5-naphthalenesulfonyl-L-lysine (DNS-lysine) was specifically bound in the combining sites of anti-DNS IgM antibodies from the horse, pig, and nurse shark. Fluorescence lifetime analysis showed the presence of a long lifetime component (21–27 ns) with antibodies from all three species. With the mammalian antibodies, the fluorophore appeared to be rigidly bound in the combining sites as judged by the presence of induced circular dichroism of DNS-lysine (equine antibodies) and single exponential anisotropy decay of the isolated Fabμ fragments (equine and porcine antibodies). The small amount of available purified nurse shark antibody did not allow preparation of fragments or induced circular dichroism measurements to directly determine rigidity of fluorophore binding. However, at least some of the hapten must have been rigidly bound since long rotational correlation times were measured for the shark DNS-lysine–anti-DNS complexes. When the emission anisotropy of the fluorophore–anti-DNS IgM complexes was measured as a function of time, it was found that all three antibody species exhibited restricted segmental flexibility in the nanosecond time range. Moreover, when the equine anti-DNS IgM was exposed to 1 M acetic acid for 1 h, the antibody underwent a conformational change which resulted in an increase in its overall flexibility. Comparison of the rotational correlation times of native equine IgM and of proteolytic fragments indicated that flexibility of IgM consists of either hindered rotation of the Fab′μ segment or a combination of at least two modes of motion: rotation of Fabμ and/or Fab′μ and bending of the entire $(Fab')_{2}\mu$ region as a unit. Similar modes of flexibility also occur in native porcine IgM. In acid exposed equine IgM, the major contribution to depolarization is from independent rotation or wagging of the Fab′μ segments. Thus, acid apparently causes a conformational change in or near the Cμ2 domains. In contrast, flexibility in nurse shark IgM appears to involve only bending of $(Fab')_{2}\mu$ as a unit. Our results suggest that segmental flexibility is an essential functional feature of all IgM antibodies and that control of this flexibility through domain interactions may play an important role in such conformationally sensitive functions as complement fixation.

The question of segmental flexibility in the immunoglobulins has been the subject of considerable attention since it was first proposed for immunoglobulin G (IgG)[1] by Noelken et al. (1965) (for reviews, see Metzger, 19 ; Cathou and Dorrington, 1975). Electron microscopic studies have shown that the Fab segments can take on different orientations with re-

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[1] Abbreviations used: DNS-lysine, ϵ-1-dimethylamino-5-naphthalenesulfonyl-L-lysine; anti-DNS, antibodies elicited to the DNS-lysine hapten which show measurable binding of the DNS determinant; DNS–HSA, DNS covalently conjugated to human serum albumin; dansic acid, dimethylaminonaphthalenesulfonic acid; KLH, keyhole limpet hemocyanin; Tris, tris(hydroxymethyl)aminomethane; 2-ME, 2-mercaptoethanol; C1q, first component of complement; IgM, immunoglobulin M; IgG, immunoglobulin G.