Cullis, P. R., & Hope, M. J. (1978) *Nature (London) 271*, 672.
Gaber, B. P., Yager, P., & Peticolas, W. L. (1978) *Biophys. J. 24*, 677.
Mabrey, S., & Sturtevant, J. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3862.
Mabrey, S., & Sturtevant, J. M. (1978) *Methods Membr. Biol. 9*, 237.
McIntosh, T. (1980) *Biophys. J. 29*, 237.
Nagle, J. F. (1976) *J. Mol. Biol. 27*, 233.
Nagle, J. F., & Wilkinson, J. A. (1978) *Biophys. J. 23*, 159.
Pauling, L. (1960) *The Nature of the Chemical Bond*, 3rd ed., Cornell University Press, Ithaca, NY.
Tardieu, A., Luzzatti, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711.
Templin, P. R. (1956) *Ind. Eng. Chem. 48*, 154.
Wilkinson, D. A., & Nagle, J. F. (1979) *Biochemistry 18*, 4244.
Yellin, N., & Levin, I. W. (1977) *Biochemistry 16*, 642.

Effects of Limited Denaturation by Heat on the Dynamic Conformation of Equine Immunoglobulin M Antibody and on Interaction with Antigen and Complement†

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ABSTRACT: In this paper, we report quantitative studies on the effects of heating equine immunoglobulin M (IgM) anti-5-(dimethylamino)naphthalene-1-sulfonyl (anti-dansyl) antibodies at 60 °C for 30 min on antigen binding and interaction with complement; parallel studies on IgM conformation and segmental flexibility were performed to localize these effects. Binding of the heated IgM to the hapten ϵ-dansyl-Lys and to the multivalent antigen dansyl$_{92}$-ficoll was measured by fluorescence enhancement and solid-phase radioimmunoassay, respectively, and found to be about half that for untreated IgM in both cases (K_0 for untreated IgM and ϵ-dansyl-Lys was 2.8×10^6 M^{-1}). Although binding of [^{125}I]C1q was only decreased 4-fold, antigen-independent complement fixation was decreased 40-fold. Antigen-enhanced complement fixation was abolished, even at IgM and antigen concentrations sufficient to compensate for reduced binding. The effects of heating on IgM conformation were determined with three independent and complementary approaches: (1) measurement of Fabμ segmental flexibility by nanosecond fluorescence depolarization; (2) circular dichroism (CD) of proteolytic fragments; and (3) binding of specific anti-Cμ_2 antibodies. Both heat-treated IgM and the (Fab′)$_2\mu$ fragment exhibited increased segmental flexibility; the latter showed the presence of a larger rotational subunit which probably includes part or all of the Cμ_2 domain. Comparison of the UV circular dichroism spectra of untreated and heated Fabμ fragments showed that only a localized change, probably in V_L, occurred on heating. In contrast, more general changes occurred in heated (Fab′)$_2\mu$ fragments. Since the only difference between Fabμ and (Fab′)$_2\mu$ is the presence of the Cμ_2 domains, the observed CD changes must have occurred in the latter. Similarly, binding measurements of specific anti-Cμ_2 antibodies to IgM showed that 2–3 times as much heated as untreated IgM was required for equivalent binding. Taken together, all of these results suggest that the conformation of the Cμ_2 domains was preferentially altered, and interactions between adjacent Cμ_2 domains within each IgM were weakened by heating the IgM. It seems reasonable, then, to implicate a structural requirement for intact, paired Cμ_2 domains in complement fixation. Since we have recently shown that optimal complement fixation occurs when several Fab's from the same IgM bind to the same antigen molecule and that a site for complement binding in addition to that for C1q can be inferred [Siegel, R. C., & Cathou, R. E. (1980b) *J. Immunol. 125*, 1910], we conclude that the role of antigen may be to stabilize an appropriate conformation of IgM for second-site binding.

Immunoglobulin M (IgM) is both the first immunoglobulin to appear in a primary immune response and the largest naturally occurring polymeric antibody with ten potential antibody binding sites (Ashman & Metzger, 1969; Metzger, 1970; Kim & Karush, 1973). Although the intrinsic affinity of each site for ligand is on the order of 10^5 M^{-1}, multivalency provides functional affinities on the order of 10^{10}–10^{11} M^{-1} (Karush, 1978). Electron micrographs of IgM bound to bacterial flagella have shown that IgM can adopt a number of different conformations of which the most striking is the staple structure, in which several Fabμ moieties are bound to the same antigenic surface (Feinstein et al., 1971). In another study, Feinstein & Munn (1969) reported that not all of the Fabμ regions of uncomplexed IgM were always seen, an observation that led them to suggest that the plane of the (Fab′)$_2\mu$ may be perpendicular to the major plane of the (Fc)$_5\mu$ ring. More recently, electron micrographs of IgM, in which significantly more detail could be seen, revealed IgM molecules with compact, asymmetric structures in which usually five Fab's radiated outward, and the other Fabμ was apparently

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tucked underneath (C. A. Smith, G. W. Seegan, R. C. Siegel, V. N. Schumaker, and R. E. Cathou, unpublished experiments). Such a conformation would be expected to be suited for binding in a multivalent fashion to bacterial and viral surfaces and might also be easily transformed into a staple structure.

The IgM μ chain contains five homology domains (Putnam et al., 1973; Watanabe et al., 1973). Compared to the γ chain, the additional domain is $C\mu_2$, which is located just above the inter-μ chain disulfide bridge (Cys-337) (Putnam et al., 1973; Watanabe et al., 1973). In a given IgM subunit, the adjacent $C\mu_2$ domains appear to be closely paired with each other (Shelton & McIntire, 1970; Parkhouse et al., 1970; Feinstein et al., 1971). There is no recognizable hinge-region sequence as is seen in γ chains (Putnam et al., 1973; Watanabe et al., 1973; Edelman et al., 1969). Nevertheless, IgM displays limited segmental flexibility (Holowka & Cathou, 1976b) similar to that observed in IgG (Yguerabide et al., 1970; Lovejoy et al., 1977), so that sequences containing multiple prolines do not appear to be necessary for this to occur.

A number of recent studies have shown the importance of $C\mu_2$ domain interactions in IgM. Limited exposure of the antibody to denaturing conditions can effect the apparent low energy of binding between domains and alter the compactness of the IgM molecule. For example, when IgM is heated to 60 °C, the molecule can now be efficiently cleaved by trypsin into Fabμ and $(Fc)_5$ fragments (Plaut & Tomasi, 1970); furthermore, if the molecule is first exposed to low concentrations of urea, heating is no longer necessary for subsequent trypsin cleavage to occur (Shimizu et al., 1974). Finally, on exposure to 1 M acetic acid, segmental flexibility is significantly increased (Holowka & Cathou, 1976b). In all these cases, changes in or near the $C\mu_2$ domains must have occurred which made this region more accessible and/or diminished for domain interactions.

Most interestingly, IgM antibodies that have been exposed to any one of these denaturants have been reported to still bind antigen but can no longer fix complement (Deutsch & Amirian, 1968; Cunniff & Stollar, 1968; Stollar et al., 1976).

We therefore felt it useful to investigate more closely the effects of such limited denaturation on IgM conformation, with a view toward determining, if possible, the changes involved and quantitating interactions of the IgM with both antigen and the complement system.

Materials and Methods

Preparation of Anti-dansyl[1]-IgM and Its Proteolytic Fragments. Equine IgM anti-dansyl antibodies were obtained and purified as described by Holowka & Cathou (1976a) and Siegel & Cathou (1980b).

$(Fab')_2\mu$ and Fabμ fragments were prepared by pepsin digestion with an enzyme/substrate ratio of 1:50 (w/w) in 0.2 M sodium acetate buffer, pH 4.5, at 37 °C for either 2.25 h [for $(Fab')_2\mu$] or 4 h (for Fabμ) (Holowka & Cathou, 1976a). The reaction was stopped by raising the pH to 8 with solid Tris base. The reaction mixture was then concentrated by ultrafiltration and fractionated on columns of Sephadex G-200 equilibrated with 0.02 M Tris–0.15 M NaCl buffer, pH 8.

The $(Fc)_5\mu$ fragment was produced by TPCK–trypsin (Worthington Biochemical Corp., Freehold, NJ) digestion at 60 °C for 30 min (Plaut & Tomasi, 1970; Zikan & Bennett, 1973). Soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) was added to inactivate the trypsin. The solution was then concentrated by ultrafiltration and fractionated on columns of either Sepharose 6B or Sepharose 4B equilibrated with 0.02 M Tris–0.50 M NaCl buffer, pH 8.

The purity of these preparations was established by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analytical ultracentrifugation. All of these preparations showed single bands upon electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate and sedimented as singly symmetrical peaks in the analytical untracentrifuge.

Heat Treatment of Anti-dansyl-IgM, $(Fab')_2\mu$, and Fabμ. Purified IgM, $(Fab')_2\mu$, and Fabμ in 0.02 M Tris–0.15 M NaCl buffer, pH 8, were heated at 60 °C for 30 min, then chilled on ice, and fractionated on columns of Sepharose 4B (IgM) or G-200 [$(Fab')_2\mu$ and Fabμ] to remove any aggregates which may have formed. Usually a maximum of 20–30% of the preparations were aggregated by heat treatment.

Preparation of C1q. Human C1q was a generous gift from Dr. D. Bing. The C1q was prepared according to the procedure of Assimeh et al. (1974) and stored at −80 °C.

Complement Assays. Microcomplement fixation was done according to the method of Wasserman & Levine (1961) as modified by Stollar (1978). The C1q binding assay was based on the ability of a soluble immunoglobulin to inhibit the binding between ^{125}I-labeled C1q and a solid phase composed of a stable immune complex and was similar to that described by Heusser et al. (1973).

Antigen and Hapten Binding Assays. The binding of the hapten dansyl-Lys (Sigma Chemical Co., St. Louis, MO) was estimated by a fluorescence enhancement assay (Holowka & Cathou, 1976a) and analyzed by the method of Scatchard (1949). Corrected fluorescence measurements were made on an Aminco SPF 1000 spectrofluorometer (American Instrument Co.) operated in a time base mode with the excitation wavelength set at 330 nm and the emission wavelength set at 480 nm.

Binding of a dansyl-ficoll to anti-dansyl-IgM antibody was estimated by using a solid-phase assay. Iodinated dansyl-ficoll was absorbed to polystyrene tubes by incubating 200 μL of a 0.5 μg/mL solution in 0.02 M Tris–0.15 M NaCl buffer, pH 8, containing 0.1% BSA for 2 h at 37 °C and then overnight at 4 °C. Before use, each tube was washed with 200 μL of 0.02 M Tris–0.15 M NaCl buffer, pH 8, containing 0.1% BSA, and counted in a γ counter. With this procedure, 33–40 ng of material would remain firmly bound to each tube, and the average amount of ^{125}I-labeled dansyl-ficoll bound varied by no more than ±3% in a given assay.

To measure binding, serial dilutions of ^{125}I-labeled anti-dansyl-IgM were added in a final volume of 25 μL to each tube. A duplicate set of tubes was set up which contained excess hapten to serve as a control. Buffer controls were also included to ensure that there was no loss of ^{125}I-labeled dansyl-ficoll from the tubes. The reaction was incubated for 60 min at 37 °C and mixed on a Vortex mixer every 10 min. The supernatants were withdrawn by aspiration and the tubes washed once with 0.02 M Tris–0.15 M NaCl buffer, pH 8, containing 0.1% BSA and, in the hapten control, buffer containing hapten. In all cases, no more than 10% of the antigen leached off the tubes during the assay. The tubes were counted, and the amount of ^{125}I-labeled IgM bound was determined. This value was corrected for nonspecific binding by using the hapten control and that for leaching of ^{125}I-labeled dansyl-ficoll by using the buffer control.

Iodination Procedures. IgM and dansyl-ficoll were iodinated with Na^{125}I (Amersham, Arlington Heights, IL) by

[1] Abbreviations used: dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; BSA, bovine serum albumin; CD, circular dichroism; TPCK, N^{α}-tosylmethylalanine chloromethyl ketone.

using the procedure of Unkeless & Eisen (1975). The specific activity of the labeled product was 3×10^5 cpm/μg; 5–10% of the IgM molecules were labeled.

C1q was iodinated by using the lactoperoxidase procedure of Heusser et al. (1973). Specific activities of (3–4) $\times 10^5$ cpm/μg were obtained; 1–2% of the C1q molecules were labeled.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by using the Tris–glycine method of Laemmli (1970). Samples were mixed with an equal volume of 2% sodium dodecyl sulfate, 0.2 M Tris, and 8 M urea, pH 8, and incubated for 2 h at 37 °C prior to electrophoresis.

In cases where reduction and alkylation were necessary, samples were reduced with 50 mM dithiothreitol (DTT) in 0.2 M Tris, 4 M urea, and 1% sodium dodecyl sulfate, pH 8, for 60 min at 37 °C and then alkylated with 120 mM iodoacetamide (Sigma Chemical Co., St. Louis, MO) for 30 min at 37 °C.

Analytical Ultracentrifugation. Sedimentation coefficients were determined with a Beckman Spinco Model E analytical ultracentrifuge equipped with schlieren optics. Protein solutions (0.5–2.0 mg/mL) were centrifuged in a 30-mm cell at 48 000 rpm at 20 °C.

Binding of Anti-Cμ_2 Antisera to IgM. The preparation of antibodies specific for the Cμ_2 domain will be described elsewhere (Siegel et al., 1980). Binding of a test substance to the anti-Cμ_2 antisera was assayed by an inhibition assay. Briefly, serial dilutions of various inhibitors were added to 0.2 μg of ^{125}I-labeled IgM and a 1:3 dilution of anti-Cμ_2 antisera (the amount needed to bind 40–60% of the added ^{125}I-labeled IgM) in a total volume of 150 μL. The reaction mixture was incubated at 35 °C for 1 h; then 50 μL of goat antirabbit IgG was added, and the reaction mixture was incubated for 1 h at 37 °C followed by overnight incubation at 4 °C. Each tube was centrifuged at 7000g for 10 min, and 150 μL of the supernatant was removed and counted in a γ counter. Controls always included tubes without anti-Cμ_2 antisera, without antirabbit IgG, without inhibitor, and with ^{125}I-labeled IgM only.

Circular Dichroism. CD measurements of anti-dansyl-IgM and the enzymatic fragments derived from it were made on a Jasco J-20 recording spectropolarimeter. Cylindrical quartz–silica cells with path lengths of 0.1–2.0 cm and protein concentrations of 0.1–1.0 mg/mL were used. The data were expressed as mean residue ellipticity ($[\theta]_\lambda$ = deg cm^2 dmol^{-1}).

Nanosecond Fluorescence Measurements. The kinetics of fluorescence decay were measured by using the monophoton counting technique (Yguerabide, 1972). The instrument was a modified Ortec 9200 that has previously been described (Holowka & Cathou, 1976b). Emission anisotropy as a function of time [$A(t)$] was calculated according to

$$A(t) = \frac{f_\parallel(t) - gf_\perp(t)}{f_\parallel(t) + g2f_\perp(t)} \quad (1)$$

where $f_\parallel(t)$ is the fluorescence intensity as a function of time of the vertically polarized emission component, and $f_\perp(t)$ is the fluorescence intensity as a function of time of the horizontally polarized emission component, and $g = F_\parallel/F_\perp$ and is the steady-state normalization ratio (Holowka & Cathou, 1976b).

The anisotropy decay data were interpreted as the sum of two major modes of Brownian motion and fit to a sum of two exponentials by using a weighted nonlinear least-squares analysis (Marquardt, 1963; Holowka & Cathou, 1976b):

$$A(t) = A_0(f_s e^{-t/\phi s} + f_L e^{-t/\phi L}) \quad (2)$$

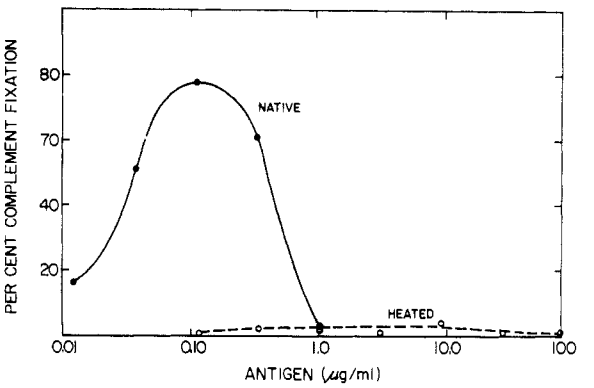

FIGURE 1: Antigen-dependent complement fixation by 0.5 μg/mL dansyl-IgM (●) and by 100 μg/mL heated anti-dansyl-IgM (○) as a function of dansyl$_{92}$-ficoll concentration.

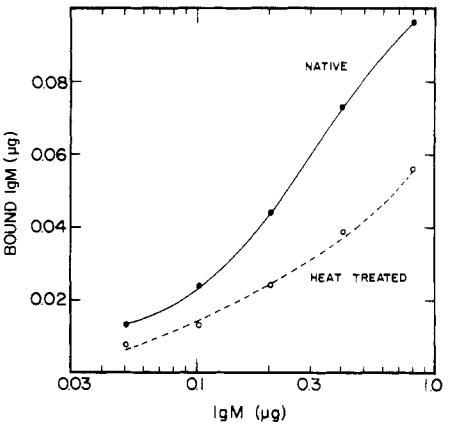

FIGURE 2: Binding of ^{125}I-labeled anti-dansyl-IgM, native (●) or heated (○) to dansyl$_{92}$-ficoll-coated plastic tubes.

The analysis is described in the Appendix.

Results

Comparison of Interactions of Native and Heat-Treated IgM with Antigen and the Complement System. Incubation of IgM at 60 °C for 30 min followed by removal of any aggregates had a dramatic effect on the ability of the IgM to fix complement. As shown in Figure 1, 0.5 μg/mL native IgM showed optimal complement fixation in the presence of 0.1 μg/mL antigen above that shown by the IgM alone, whereas even 100 μg/mL heated IgM showed no enhanced complement fixation over a wide range of antigen concentrations.

This effect was partially reversible. Incubation of the antibody at 4 °C for 2–4 weeks resulted in recovery of up to 50% of the original complement-fixing activity (data not shown), indicating that heat treatment had not selected a non-complement-fixing population of antibody.

Heated IgM, however, could still fix complement in the absence of antigen, although higher concentrations were necessary. An aliquot of 1 μg/mL native IgM fixed 50% complement whereas 40 μg/mL heated IgM was required. The (Fc)$_5$ fragment produced by trypsin cleavage (which is exposed to heat prior to proteolysis) was about as active as whole heated IgM, i.e., 25 μg/mL was necessary for 50% complement fixation.

Only part of this difference in complement fixation by IgM was due to an alteration of C1q binding: 120 μg/mL heated IgM vs. 32.5 μg/mL native IgM was necessary for 50% inhibition of C1q binding in the competitive assay, i.e., a 4-fold reduction.

Experiments designed to measure the binding of native and heated IgM to the hapten ϵ-dansyllysine are illustrated in Table

Table I: Binding of Anti-dansyl-IgM and Its Fabμ Fragments to ε-Dansyllysine[a]

	$K_0 \times 10^{-6}$	n[b]
native IgM	2.8 ± 1.1	5.1 ± 1.0
heat-treated IgM[c]	4.6 ± 3.7	4.7 ± 0.5
native Fabμ	2.8 ± 0.4	0.34 ± 0.01
heat-treated Fabμ[c]	1.7 ± 0.1	0.39 ± 0.01

[a] Measured by fluorescence enhancement of ε-dansyllysine; see Materials and Methods. [b] Number of binding sites per mol of IgM or Fabμ. [c] Heated at 60 °C for 30 min and then purified; see Materials and Methods.

I and Figure 2. Heated IgM appeared to bind hapten with a similar affinity as that of the native IgM. However, binding experiments with the respective Fab fragments, which showed considerably lower statistical variation, yielded a 60% lower binding constant upon heating. This pattern was borne out when binding to the multivalent antigen dansyl$_{92}$-ficoll was measured. As shown in Figure 2, 2–3 times as much heated IgM as native IgM was required to bind an equivalent amount of antibody.

When all of these factors were taken into account, i.e., the affinity of heated IgM for antigen appeared to be about half that of native IgM, antigen-independent complement fixation had decreased by a factor of 40, and appropriate concentrations of antibody and antigen were employed to provide optimal conditions for binding of all of the components, it was found, nevertheless, that there was no antigen-enhanced complement fixation. These results suggest, then, that the role of antigen in the antibody–antigen complex is negated by heating the IgM. The effects of heating on IgM conformation were investigated to gain further insight into the possible nature of such a role.

Studies on IgM Conformation. Several approaches were employed to determine the effects of heating on IgM conformation. These included (1) measurement of segmental flexibility by nanosecond fluorescence depolarization, (2) comparison of the CD spectra of IgM and its proteolytic fragments (those obtainable), and (3) binding of specific anti-Cμ_2 antibodies. These experiments were designed to determine which domains of IgM were primarily affected by the heat treatment.

Segmental Flexibility. Figure 3 shows a comparison of the time-dependent anisotropy of native and heated IgM. The decay exhibited by native IgM was identical with that observed earlier by Holowka & Cathou (1976b). The faster decay exhibited by heated IgM can be interpreted in terms of increased internal flexibility (Holowka & Cathou, 1976b; Harvey & Cheung, 1980). When the data were fit to a sum of two exponentials (Holowka & Cathou, 1976b), the parameters shown in Table II were obtained. While heat treatment did not significantly alter the value of the shorter correlation time, it did change the contribution to total depolarization from 49 to 62%; similarly, the value of the longer correlation time was not altered, but the contribution was correspondingly decreased. It should be noted that the values

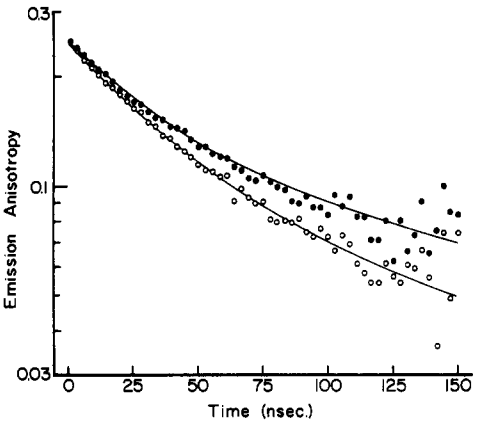

FIGURE 3: Time-dependent emission anisotropy of dansyl-Lys–anti-dansyl-IgM complexes: native IgM (●); heated IgM (○). The solid curves are the weighted nonlinear least-squares fit of the observed data to the equation $A(t) = A_0[f_s e^{-t/\phi s} + f_L e^{-t/\phi L}]$. The parameters are given in Table II.

for native IgM reported in Table II are somewhat different from those reported for similar preparations by Holowka & Cathou (1976b). A reexamination of their data by using the newer weighted nonlinear least-squares analysis described in the Appendix resulted in similar parameters to those shown in Table II. The apparent difference can be ascribed to different weights used in the earlier and present analyses: we believe that our present procedure, which weights each datum by a term that is inversely proportional to the variance of the datum, rather than the anisotropy, is more accurate and reproducible (Bevington, 1969).

The anisotropy decays of native and heated (Fab′)$_2\mu$ fragments were qualitatively similar to those exhibited by IgM. Comparison of the parameters showed the same trends seen with IgM, but significant differences were also now apparent in the short correlation times. The value of ϕ_s was increased from 22 to 30 ns, and the value of the weighting factor doubled. At the same time, the value of ϕ_L was hardly changed. These results suggest that heated (Fab′)$_2\mu$ exhibited significantly more segmental flexibility and that the average size of the rotational subunit was somewhat increased.

Circular Dichroism of IgM and Proteolytic Fragments. The CD spectra of native and heat-treated IgM, as well as those of Fabμ and (Fab′)$_2\mu$ fragments, were examined to localize the conformational changes produced by heating. Although it would have been highly desirable to also compare the spectra of native and heated (Fc)$_5\mu$, especially since this fragment is thought to contain the C1 binding site, the unavailability of native (Fc)$_5\mu$ ruled out this comparison.

The CD spectra of native and heated IgM are compared in Figure 4. The spectrum of native IgM is that of a typical immunoglobulin, with a negative band at 217 nm, and with several bands in the aromatic region above 260 nm; little fine structure is evident except that the aromatic bands between 260 and 280 nm are negative while those above 280 nm are positive. In the spectrum of the heated IgM, the band at 217

Table II: Time-Dependent Fluorescence Anisotropy of IgM and (Fab′)$_2\mu$[a]

	A_0 ± SD	t_{max} (ns)[b]	f_s ± SD	ϕ_s (ns) ± SD	f_L ± SD	ϕ_L (ns) ± SD
native IgM	0.248 ± 0.002	150	0.49 ± 0.01	34 ± 2	0.51 ± 0.01	241 ± 7
heat-treated IgM[c]	0.246 ± 0.002	150	0.62 ± 0.08	39 ± 7	0.38 ± 0.08	243 ± 76
native (Fab′)$_2\mu$	0.254 ± 0.005	115	0.30 ± 0.07	22 ± 4	0.70 ± 0.07	85 ± 9
heat-treated (Fab′)$_2\mu$[c]	0.250 ± 0.005	115	0.60 ± 0.07	30 ± 4	0.40 ± 0.07	93 ± 11

[a] Summary of the parameters obtained by fitting the anisotropy to a sum of two exponentials $[A(t) = A_0(f_s e^{-t/\phi s} + f_L e^{-t/\phi L})]$ by a weighted nonlinear least-squares analysis. [b] Longest time to which $A(t)$ data points were fit. [c] Heated at 60 °C for 30 min and then purified; see Materials and Methods.

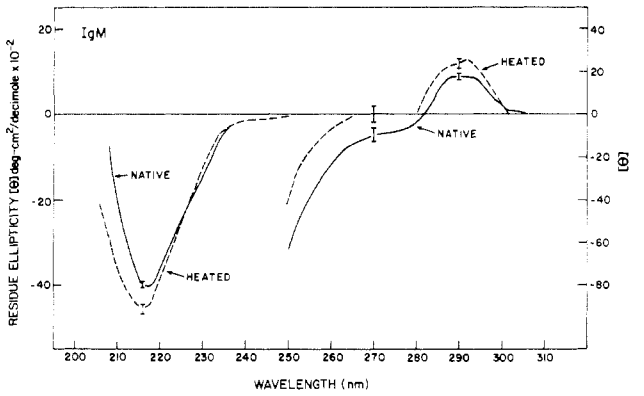

FIGURE 4: CD spectra of native anti-dansyl-IGM (—) and heated anti-dansyl-IgM (- - -).

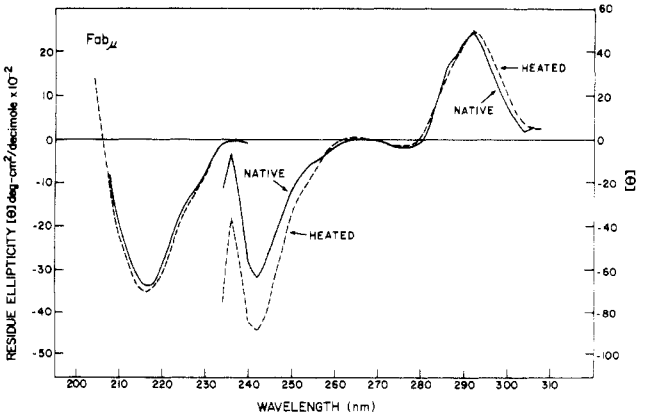

FIGURE 5: CD spectra of native Fabμ (—) and heated Fabμ (- - -) fragments of anti-dansyl-IgM.

nm was increased by 13% and shifted to 216 nm. In the aromatic residue region, the negative bands were eliminated, and the positive bands were enhanced by 34%. A positive band at 292 nm was also partially resolved.

In contrast, the CD spectra of native and heated Fabμ fragments were very similar to each other (see Figure 5). In both cases, there was a negative band at 216 nm and positive ellipticity between 280 and 305 nm. Native Fabμ exhibited a poorly resolved shoulder at 286–288 nm that was lost on heating. The major difference was in the magnitude of a positive band at 235 nm, a band which has been ascribed in other immunoglobulins to a tyrosine (Cathou et al., 1968). It would appear that any changes in conformation of Fabμ produced by heating must have been minor.

Differences in the CD spectra of native and heated $(Fab')_2\mu$ were more marked. As shown in Figure 6, the CD spectrum of native $(Fab')_2\mu$ could be distinguished from that of native Fabμ primarily in the region above 250 nm. The CD spectrum of $(Fab')_2\mu$ exhibited a negative band centered at 280 nm, which was not present in the Fabμ spectrum to any significant extent, and the positive bands centered at 290 nm were much smaller. When $(Fab')_2\mu$ was heated, changes were observed in all parts of the spectrum. Since $(Fab')_2\mu$ contains $C\mu_2$ while Fabμ does not, these spectral differences between $(Fab')_2\mu$ and Fabμ must reflect conformational changes in $C\mu_2$ and/or interactions of $C\mu_2$ with $C\mu_1$.

Anti-$C\mu_2$ Antibodies. Antiserum specific for the $C\mu_2$ region of IgM was reacted with native and heated IgM. The results are shown in Figure 7. It can be seen that while the antiserum bound to both preparations, 2–3 times as much heated as native IgM was required for equivalent binding, which suggests that the conformation of the $C\mu_2$ domains was altered on heating.

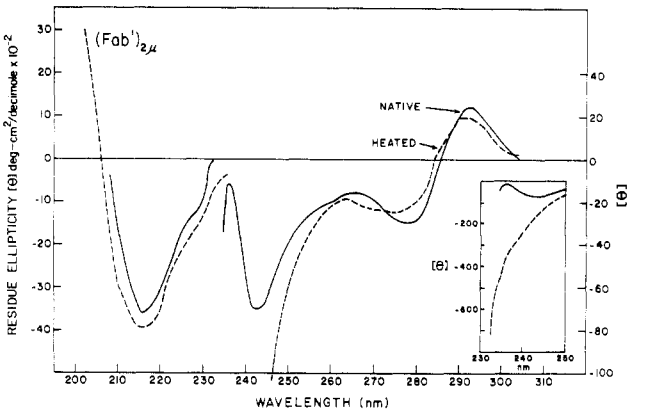

FIGURE 6: CD spectra of native $(Fab')_2\mu$ (—) and heated (- - -) $(Fab')_2\mu$ fragments of anti-dansyl-IgM.

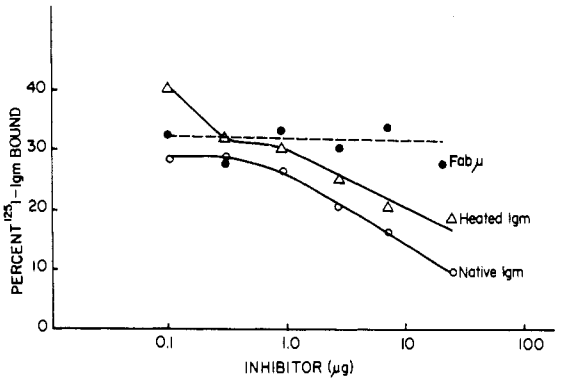

FIGURE 7: Binding of anti-$C\mu_2$ antibodies to native anti-dansyl-IgM (○), heated anti-dansyl-IgM (△), and Fabμ fragments (●) in an inhibition assay. (For details of the assay, see Materials and Methods.)

Discussion

Effect of Heating on IgM Conformation. The value of ϕ_s, the short correlation time observed for native $(Fab')_2\mu$ (22 ns), was very similar to that observed for intact rabbit IgG (24–26 ns) (Chan & Cathou, 1977) so that it is reasonable to assume that Fabμ is the smallest rotational subunit and that some segmental flexibility occurs between Fabμ and $C\mu_2$. When the $(Fab')_2\mu$ fragment is heated, the value of ϕ_s was increased to 30 ns, which suggests contributions from a larger rotational subunit, and the contribution to anisotropy decay, f_s, was increased dramatically to 0.60 from 0.30. The value of f_s is dependent on bending motion, such as movement of Fabμ and/or Fab'μ segments within the molecule, so that an increase in its value for a system that has been perturbed primarily by a change in hinge point probably monitors a change in segmental flexibility, in this case an increase. However, since the types of motions are unknown (i.e., they could occur in several planes), the range of bend angles in both native angles in both native and heated $(Fab')_2\mu$ remains unknown (Harvey & Cheung, 1980). Nevertheless, the simplest interpretation of the increased values of ϕ_s and f_s observed for heated $(Fab')_2\mu$ is that interactions between the adjacent $C\mu_2$ domains have decreased.

This situation is analogous to that which occurs on reduction and alkylation of the single interchain disulfide at the hinge of rabbit IgG: both ϕ_s and f_s increase (Chan & Cathou, 1977). Romans et al. (1977) have observed that on reduction and alkylation of some human IgG molecules the latter agglutinates more readily with cellular antigens, an observation which they interpreted to indicate greater freedom of motion of the Fab regions. The electron microscopic studies of Seegan et al. (1979) have also shown that reduction and alkylation of IgG cause the Fab length to increase to encompass the C_{H^2} domain.

The larger value of ϕ_s observed in native IgM compared to that for $(Fab')_2\mu$ is most likely due to contributions of a larger rotational subunit, most likely $(Fab')_2\mu$ (Holowka & Cathou, 1976b). Since analysis of the time-dependent anisotropy of a larger molecule such as IgM with only two exponential components is undoubtedly an oversimplification, ϕ_s for IgM probably represents an average of several modes of motion. Furthermore, $(Fab')_2\mu$ in IgM has fewer degrees of freedom since it is attached to Fc. At the same time, the value of ϕ_L, 241 ns, is shorter than that expected for the tumbling motion of the whole IgM molecule even if it were a rigid hydrated sphere (380 ns), so internal flexibility may exist in $(Fc)_5\mu$ as well. All of these considerations make it difficult to describe the motion of IgM in simple terms. Nevertheless, the trend in ϕ_s and f_s observed on heating IgM is the same as that observed in the simpler $(Fab')_2\mu$ fragment, so it seems reasonable to assume that adjacent $C\mu_2$ domains become unpaired in the whole molecule as well.

The most prominent change in the CD spectrum of Fab_μ on heating was in the reduction of the positive band at 235 nm, which is probably due to tyrosine (Cathou et al., 1968). Although the locations of optically active tyrosine residues in equine Fabμ are unknown, since the affinities for hapten and antigen on heating were also decreased, the 235-nm band is probably due to one or more tyrosines in the variable domains. Equine light chains are 100% λ (Hood & Prahl, 1971); the variable, but not the constant, domain of human λ chain also displays a 235-nm band (Björk et al., 1971); by analogy, then, the 235-nm band in Fabμ may arise in V_L. Since essentially no change was observed in the contributions from backbone folding, the conformational change in Fabμ is probably localized to the variable region.

In contrast, the more marked changes observed in the CD spectrum of heated $(Fab')_2\mu$, which occurred throughout the spectrum, can be ascribed to a conformational change within the $C\mu_2$ domain and/or domain–domain interactions between adjacent $C\mu_2$'s. Such a change in $C\mu_2$ conformation was confirmed by the decreased binding to anti-$C\mu_2$ antibodies.

Finally, on quantitative comparison of the changes in CD observed for whole IgM and $(Fab')_2\mu$, it can be seen that additional changes have occurred in IgM which may be due to changes in $(Fc)_5\mu$: the increase in the 217-nm band in heated IgM is about 25% larger than that observed in $(Fab')_2\mu$, and there is additional loss of a negative contribution at 290 nm. We are currently trying to produce an isolated $(Fc)_5\mu$ fragment from IgM that has not been exposed to denaturing agents to confirm this possibility.

Role of Antigen in Complement Fixation. When IgM is heated, binding affinity to hapten and antigen was reduced to 60% of its original value. Similarly, binding to C1q was reduced to 25%. Antigen-independent fixation of complement was reduced further to 2.5% and affected all of the IgM molecules rather than partial inactivation of a subpopulation (Siegel, 1980). Similarly, in preliminary experiments on antigen-independent C1 activation, 1 μg of native IgM was fully active while 20 μg of heated IgM was inactive, indicating a reduction of activity to 5% or lower (N. Cooper, R. C. Siegel, and R. E. Cathou, unpublished experiments). Since complement fixation reflects both binding and activation of C1, as well as possible interactions of immunoglobulin with other complement components (Fewtrell et al., 1979), these results suggest a more complex interaction of IgM and complement exists then that seen with isolated C1q (see below).

However, if sufficient antibody, antigen, and complement were provided to compensate for these reductions in affinity, the heated IgM–antigen complexes showed only the same complement fixation as that exhibited by heated IgM alone, rather than enhancement in the presence of antigen. Thus, the normal role of antigen was apparently eliminated, even though antibody–antigen complexes were still formed.

Two major roles can be considered for antigens in complement fixation: (1) the formation of IgM aggregates to provide multivalent binding sites for multivalent C1 subcomponents or (2) stabilization of appropriate conformational changes in the IgM molecule which lead to the formation of new sites (Metzger, 1974).

The formation of IgM aggregates, while it undoubtedly occurs, is probably not the primary and sole function of antigen for several reasons. First, an aggregated fraction of heated IgM, which eluted in the void volume during Sepharose 4B chromatography, behaved identically with separated nonaggregated heat-treated IgM with respect to complement fixation. Second, small antigens precipitate with IgM antibody but do not fix complement (Siegel & Cathou, 1980b). Therefore, aggregation, per se, is insufficient.

On the other hand, the possibility of conformational changes or, more likely, the stabilization of a particular conformation in a flexible molecule such as IgM must be seriously considered. Brown & Koshland (1975) and Chiang & Koshland (1979) have proposed that univalent binding of small antigens to rabbit IgM causes complement fixation by way of an allosteric conformational change. This particular mechanism does not appear to be significant in our system with equine IgM since monovalent binding does not cause complement fixation in our hands; rather, since conversion by polymerization of a small antigen to one large enough to span the distance between neighboring Fab's does cause complement fixation, intramolecular multivalent binding of IgM appears to be important (Siegel & Cathou, 1980b). Karush et al. (1979) have recently shown that a complex between equine IgM and a divalent ligand with a potential separation of reactive groups of 20 nm will fix complement and that no significant cross-linking of IgM molecules need occur (Karush et al., 1979). Multivalent binding of several Fab's within an IgM to an antigenic surface would be expected to limit flexibility; it might also stabilize one or more conformations which would be conducive to complement binding and activation (Feinstein et al., 1971). Since C1q binding is not altered when antigen is added, it seems more likely that antigen affects interaction of another C1 subcomponent, or C4, with a second site on IgM (Siegel & Cathou, 1980b). Such interactions have been implicated in the activation of complement by IgG (Allen & Isliker, 1974; Goers & Porter, 1978; Dodds & Porter, 1979). We would postulate, then, that the altered $C\mu_2$ domains in heated IgM no longer provide stabilization for such conformations. It is also possible that the postulated second site [either on $C\mu_2$ or $(Fc)_5\mu$] is itself altered on heating. Clearly, the next step is to try to identify such second-site interactions more directly.

Appendix

The time-dependent emission anisotropy data were fit by minimizing the square of the differences between the experimental and calculated curves multiplied by the weighting factor, w_i, associated with each data point.

The weighting factor is given by (Bevington, 1969)

$$w_i = \frac{1/\sigma_i^2}{1/N\sum(1/\sigma_i^2)} \qquad \text{(A1)}$$

where w_i is the weight associated with the ith data point, σ_i^2 is the variance of the ith data point, and N is the total number

of data points. The total number of data points (N) was usually 250–350. The variance of the anisotropy, $\sigma_{A_i}^2$, is (Bevington, 1969)

$$\sigma_{A_i}^2 = \sigma_{\parallel_i}^2\left(\frac{\partial A_i}{\partial \sigma_{\parallel_i}}\right)^2 + \sigma_{\perp_i}^2\left(\frac{\partial A_i}{\partial \sigma_{\perp}}\right)^2 \quad \text{(A2)}$$

where $\sigma_{\parallel_i}^2$ is the variance of ith data point in the $f_{\parallel}(t)$ decay, and $\sigma_{\perp_i}^2$ is the variance of the ith data point in the $f_{\perp}(t)$ decay. σ_i^2 is equal to the number of counts associated with each channel (Bevington, 1969); therefore, by using eq A1 and A2, it can be shown that

$$\sigma_i^2 = \sigma_{A_i}^2 = \frac{9[f_{\perp_i}(t)][f_{\parallel_i}(t)][f_{\perp_i}(t) + g^2 f_{\parallel_i}(t)]}{[f_{\parallel_i}(t) + g2f_{\perp_i}(t)]^4} \quad \text{(A3)}$$

References

Allen, R., & Isliker, H. (1974) *Immunochemistry 11*, 243.

Ashman, R. F., & Metzger, H. (1969) *J. Biol. Chem. 244*, 3405.

Assimeh, S. N., Bing, D. H., & Painter, R. H. (1974) *J. Immunol. 113*, 225.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Björk, I., Karlsson, F. A., & Berggard, I. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1707.

Brown, J. S., & Koshland, M. E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 5111.

Cathou, R. E., Kulczycki, A., Jr., & Haber, E. (1968) *Biochemistry 7*, 3958.

Chan, M. L., & Cathou, R. E. (1977) *J. Mol. Biol. 112*, 653.

Chiang, H. C., & Koshland, M. E. (1979) *J. Biol. Chem. 254*, 2736.

Cunniff, R. V. H., & Stollar, B. D. (1968) *J. Immunol. 100*, 7.

Deutsch, G., & Amirian, K. (1968) *Immunology 15*, 623.

Dodds, A. W., & Porter, R. R. (1979) *Mol. Immunol. 16*, 1059.

Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. O., Rutishauser, V., & Waxdal, M. J. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 78.

Feinstein, A., & Munn, E. A. (1969) *Nature (London) 224*, 1307.

Feinstein, A., Munn, E. A., & Richardson, N. E. (1971) *Ann. N.Y. Acad. Sci. 190*, 104.

Fewtrell, C., Geier, M., Goetze, A., Holowka, D., Isenmen, D. E., Jones, J. F., Metzger, H., Navia, M., Sieckmann, D., Silverton, E., & Stein, K. (1979) *Mol. Immunol. 16*, 741.

Goers, J. W. F., & Porter, R. R. (1978) *Biochem. J. 175*, 675.

Harvey, S. C., & Cheung, H. C. (1980) *Biopolymers 19*, 913.

Heusser, C., Boseman, M., Nordin, J. H., & Isliker, H. (1973) *J. Immunol. 110*, 820.

Holowka, D. A., & Cathou, R. E. (1976a) *Biochemistry 15*, 3373.

Holowka, D. A., & Cathou, R. E. (1976b) *Biochemistry 15*, 3379.

Hood, L., & Prahl, J. (1971) *Adv. Immunol. 14*, 291.

Karush, F. (1978) *Compr. Immunol. 5*, 85

Karush, F., Chua, M.-M., & Rodwell, J. D. (1979) *Biochemistry 18*, 2226.

Kim, Y. D., & Karush, F. (1973) *Immunochemistry 10*, 365.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Lovejoy, C., Holowka, D. A., & Cathou, R. E. (1977) *Biochemistry 16*, 3668.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431.

Metzger, H. (1970) *Adv. Immunol. 12*, 57.

Metzger, H. (1974) *Adv. Immunol. 18*, 169.

Parkhouse, R. M. E., Askonas, B. A., & Dourmashkin, R. R. (1970) *Immunology 18*, 575.

Plaut, A. G., & Tomasi, T. B., Jr. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 318.

Putnam, F. W., Florent, G., Paul, C., Shinoda, T., & Shimizu, A. (1973) *Science (Washington, D.C.) 182*, 287.

Romans, D. G., Tilley, C. A., Crookton, M. C., Falk, R. E., & Dorrington, K. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2531.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.

Seegan, G. W., Smith, C. A., & Schumaker, V. N. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 907.

Shelton, E., & McIntire, K. R. (1970) *J. Mol. Biol. 47*, 595.

Shimizu, A., Watanabe, S., Yamamura, Y., & Putnam, F. W. (1974) *Immunochemistry 11*, 719.

Siegel, R. C. (1980) Ph.D. Thesis, Tufts University, Medford, MA.

Siegel, R. C., & Cathou, R. E. (1978a) *J. Immunol. 120*, 1798.

Siegel, R. C., & Cathou, R. E. (1978b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1853A.

Siegel, R. C., & Cathou, R. E. (1979) *Abstracts of the American Society for Photobiology Meeting*, p 171, Asilomar, CA.

Siegel, R. C., & Cathou, R. E. (1980a) *Biophys. J. 32*, 638.

Siegel, R. C., & Cathou, R. E. (1980b) *J. Immunol. 125*, 1910.

Siegel, R. C., DeGuilio, P. A., & Cathou, R. E. (1980) *Mol. Immunol.* (in press).

Stollar, B. D. (1978) *Methods Cell Biol. 18*, 105.

Stollar, B. D., Stadecker, M. J., & Morecki, S. (1976) *J. Immunol. 117*, 1387.

Unkeless, J. C., & Eisen, H. N. (1975) *J. Exp. Med. 142*, 1520.

Wasserman, E., & Levine, L. (1961) *J. Immunol. 87*, 290.

Watanabe, E., Barnikol, H. V., Horn, J., & Hilschmann, N. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 324*, 1505.

Yguerabide, J. (1972) *Methods Enzymol. 26*, 498.

Yguerabide, J., Epstein, H. F., & Stryer, L. (1970) *J. Mol. Biol. 51*, 573.

Zikan, J., & Bennett, J. C. (1973) *Eur. J. Immunol. 3*, 415.