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Detection of a Conformational Change in an Antihapten–Antibody System upon Interaction with Divalent Hapten*

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ABSTRACT: In this communication we use the technique of differential sedimentation to determine if a conformational change occurs in the antibody molecule upon interaction with hapten. We can detect any change in sedimentation coefficient greater than 0.016 S. Purified anti-2,4-dinitrophenyl antibody from rabbits was interacted with the two monovalent haptens, 2,4-dinitrophenylglycine and ε-2,4-dinitrophenyllysine, and with the two divalent haptens, α,ε-2,4-dinitrophenyllysine and 2,4-dinitrophenylcystine. Interaction with both monovalent haptens caused the hapten to undergo a bathochromic shift and a *decrease* in extinction coefficient, while interaction with both divalent haptens caused a bathochromic shift and an *increase* in extinction coefficient. Interaction with the monovalent haptens caused no change in sedimentation coefficient of the antibody molecule. The interpretation is that no detectable conformational change occurs upon interaction with monovalent hapten. Interaction with divalent hapten resulted in the formation of monomer and dimer exclusively. The monomer and dimer can be separated in the ultracentrifuge and by Sephadex G-200 chromatography with the isolated fractions remaining stable up to 2 months at 4°. The monomer showed an increase in sedimentation coefficient which corresponds to a 1% change in the antibody frictional coefficient molecule.

The technique of differential sedimentation (Schumaker and Adams, 1968) may be used to detect conformational changes in protein molecules (Schumaker, 1968). In this communication we report an attempt to use this technique to detect a conformational change in an antihapten–antibody molecule upon interaction with hapten. The system we chose to study was rabbit anti-DNP antibody.

Kinetic studies (Day *et al.*, 1963; Froese and Sehon, 1965) indicate that the rate of combination of antibody and hapten is diffusion controlled and governed by a second-order rate constant. The small activation energy of about 4 kcal/mole (Day *et al.*, 1963) has been cited as evidence that little or no conformational change occurs upon interaction of hapten with antibody. However, recent evidence (Dandliker and Levison, 1967; Levison *et al.*, 1968) seems to show that the activation energy of the second-order bimolecular reaction between antibody and a protein antigen is about 12 kcal/mole. This implies that some sort of structural changes occur in the antibody molecule prior to or during combination with antigen.

It has been found (Cathou and Haber, 1967), by the use of optical rotatory dispersion, that the binding of hapten to antibody seems to stabilize the tertiary structure of the antibody molecule. When free antibody is put into 4 M guanidine-HCl, the optical rotatory dispersion spectrum indicates unfolding of the molecule. However, in the presence of hapten, less unfolding of the molecule occurs. But these data do not indicate whether the active site is formed before or during hapten binding.

Based on hydrodynamic data, it has been proposed that the overall shape of the antibody molecule is a Y (Noelken *et al.*, 1965). This seems to be borne out by recent electron micrographs on both antigen–antibody (Feinstein and Rowe, 1965) and hapten–antibody systems (Valentine and Green, 1967). The interpretation is that the antibody molecule "clicks open" during reaction with antigen or hapten, with larger antigens producing a greater opening of the Y than smaller antigens.

If such a conformational change were to occur, one might expect it to change the frictional coefficient, and hence, the sedimentation coefficient of the antibody molecule. Our tech-

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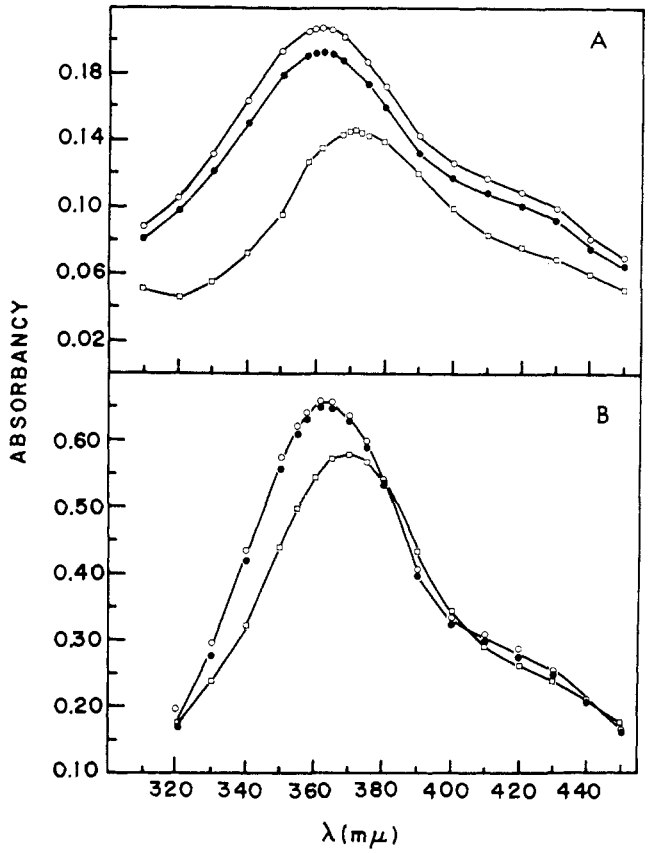

FIGURE 1: Absorption spectra of monovalent haptens. Graph A is for DNP-glycine (1.00×10^{-5} M) in 0.15 M NaCl (pH 7.0) (●), in the same solvent containing 1.79 mg/ml of IgG (○), and in the same solvent containing 1.35 mg/ml of purified rabbit anti-DNP antibody (□). Maximum absorption for free DNP-glycine and DNP-glycine in the presence of IgG occurs at 362 mμ while maximum absorption for bound DNP-glycine occurs at 372 mμ. Graph B is for ε-DNP-lysine (3.75×10^{-5} M) in 0.15 M NaCl (pH 7.0) (●), in the same solvent containing 2.36 mg/ml of IgG (○), and in the same solvent containing 2.82 mg/ml of purified rabbit anti-DNP antibody (□). Maximum absorption for free ε-DNP-lysine and ε-DNP-lysine in the presence of IgG occurs at 363 mμ while maximum absorption for bound ε-DNP-lysine occurs at 370 mμ.

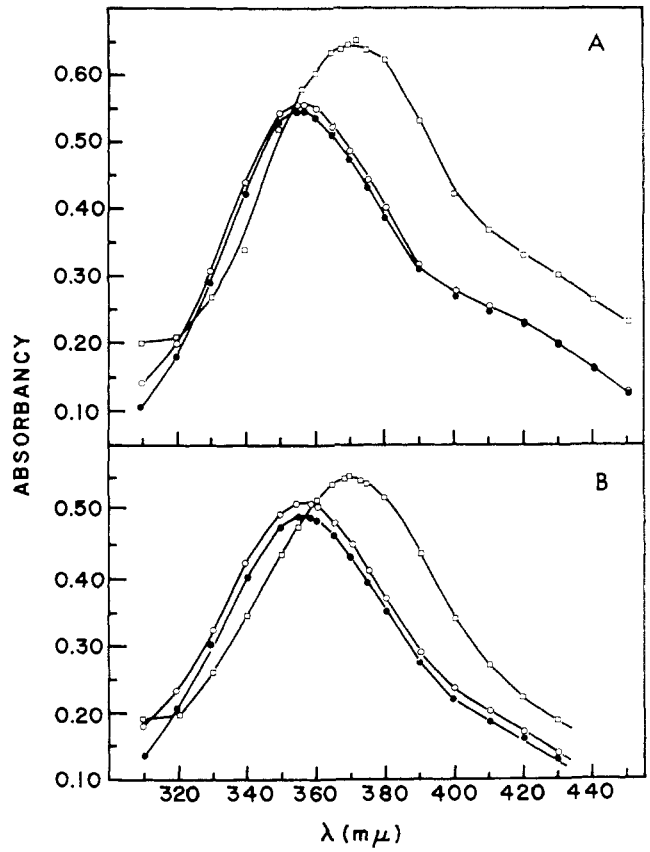

FIGURE 2: Absorption spectra of divalent haptens. Graph A is for α, ε-DNP-lysine (1.91×10^{-5} M) in 0.15 M NaCl (pH 7.0) (●), in the same solvent containing 3.69 mg/ml of IgG (○), and in the same solvent containing 2.86 mg/ml of purified rabbit anti-DNP antibody (□). Maximum absorption for free α, ε-DNP-lysine and α, ε-DNP-lysine in the presence of IgG occurs at 355 mμ while maximum absorption for bound α, ε-DNP-lysine occurs at 372 mμ. Graph B is for di-DNP-cystine (1.88×10^{-5} M) in 0.15 M NaCl (pH 7.0) (●), in the same solvent containing 2.36 mg/ml of IgG (○), and in the same solvent containing 2.82 mg/ml of purified rabbit anti-DNP antibody (□). Maximum absorption for free di-DNP-cystine and di-DNP-cystine in the presence of IgG occurs at 355 mμ while maximum absorption for bound di-DNP-cystine occurs at 370 mμ.

nique of differential sedimentation can measure differences in sedimentation coefficient of ±0.016 S. Thus, we should be able to detect any conformational change which occurs, upon interaction of antibody with hapten, if it is reflected by a change in sedimentation coefficient greater than 0.016 S.

Materials and Methods

Immunization. DNP-BγG[1] was prepared in the usual manner (Eisen *et al.*, 1953, 1959) to give 50.1 DNP groups/molecule of BγG (Eisen *et al.*, 1954). The DNP-BγG, in 0.15 M NaCl–0.02 M sodium phosphate (pH 7.4), was emulsified with an equal volume of Freund's H 37 Ra adjuvant. Each rabbit received a total of 5 mg of DNP-BγG in 1.6 ml of emulsion, *i.e.*, 0.4 ml of the emulsion was injected into each of the four footpads (Eisen *et al.*, 1959). Blood was obtained by cardiac puncture on days 41, 42, and 44, and the sera were pooled.

Precipitin Analyses. The concentration of anti-DNP antibody in the pooled antisera was determined by precipitin reactions using the heterologous antigen DNP-HSA (Eisen *et al.*, 1954). The extinction coefficient of the DNP-HSA was determined by a combination of the ultraviolet absorption spectrum and the protein concentration was determined by the method of Lowry (Lowry *et al.*, 1951). Then, the method of Eisen (Eisen *et al.*, 1954) was used to calculate the concentration of antibody from the optical density at 290 and 360 mμ of the precipitate dissolved in 0.1 N NaOH.

Purification of Antibody. The antibody from the pooled sera was purified essentially according to Eisen (Eisen *et al.*, 1967). DNP-HSA was used for the initial precipitation of antibody and DNP-glycine was the hapten used to dissolve the precipitate. (All DNP amino acids used were DNP-L-amino acids from Cyclo Chemical Co., Los Angeles, Calif.) The purified antibody was lyophilized and stored at 4°. After

[1] Abbreviations used are: BγG, bovine γ-globulin; HSA, human serum albumin; IgG, rabbit immunoglobulin G.

TABLE I: $\delta s^0_{20,w}$ of Purified DNP Antibody Compared with IgG.

Run	$\delta s^0_{20,w}$ (S)[a]
816	-0.039 ± 0.005
829	-0.041 ± 0.012

[a] We define a negative sign as meaning that the experimental protein has a smaller sedimentation coefficient than the reference IgG, while a positive sign means the reverse.

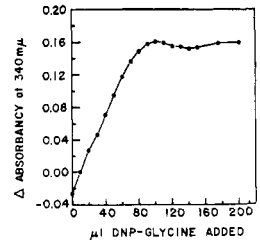

FIGURE 3: Titration of anti-DNP antibody with DNP-glycine. The absorbance of IgG at 340 mμ minus the absorbance of anti-DNP antibody at 340 mμ is plotted against the amount of DNP-glycine added. The equivalence point is at 100 μl of added DNP-glycine.

lyophilization, the yield of purified antibody was 47%. Before use, the DNP antibody was dialyzed overnight against several changes of 0.15 M NaCl (pH 7.0).

Purity and Reactivity. Evidence of purity of the antibody was obtained from the schlieren pattern, the ultraviolet spectrum, and the optical density at 360 mμ which enables us to calculate the per cent residual bound DNP-glycine. Unless otherwise indicated, the solvent used in all experiments was 0.15 M NaCl (pH 7.0).

Proof of reactivity of the antibody was obtained from spectral shifts observed upon reaction with hapten (Eisen *et al.*, 1964). Four haptens were used in this study: DNP-glycine and ε-DNP-lysine were used as monovalent haptens, while α,ε-DNP-lysine and di-DNP-cystine were used as divalent haptens. The spectral shift was measured in a Gilford spectrophotometer when equivalent amounts of antibody and hapten were present, *i.e.*, there was an available haptenic group for each available antibody combining site (assuming two combining sites per antibody molecule).

To show the percentage of antibody which interacted with hapten, we did a titration of the antibody solution. Increments of 10 μl of hapten were added directly to 1.0 ml of antibody solution in a cuvet, so that after addition of 100 μl of hapten, all antibody sites could be saturated. As controls, the same additions were made to 1.0 ml of 0.15 M NaCl solution and 1.0 ml of IgG solution. The absorbance of each was measured at 340 mμ after each addition of hapten.

IgG Immunoglobulins. IgG was either prepared from rabbit blood by a Na_2SO_4 precipitation (Kekwick, 1940) or purchased from Pentex, Inc., Kankakee, Ill. The sedimentation coefficients, as determined by differential sedimentation (see below) are identical within experimental error, so the two preparations may be used interchangeably as reference solutions in sedimentation analysis. Before use, the IgG was dialyzed overnight against 0.15 M NaCl (pH 7.0) and then either centrifuged or put through a Millipore filter to remove any turbidity.

Determination of Extinction Coefficient. The extinction coefficient of the purified anti-DNP antibody was determined by the combined use of the Brice-Phoenix differential refractometer and optical density measurements at 280 mμ using 1-mm path-length cuvets. We assumed the specific refractive index increment was the same for the purified antibody and for the IgG immunoglobulins. We measured the refractive index of a solution of IgG. The solution was then removed from the refractometer and put directly into the cuvet. The optical density at 280 mμ combined with the extinction coefficient of $\epsilon = 1.36$ ml/mg (Small and Lamm, 1966) gave the concentration of IgG directly and thus the specific refractive index increment. The same measurements were repeated for the DNP antibody preparation and, using the previously determined specific refractive index increment, the extinction coefficient was calculated.

Differential Sedimentation. This technique, as described in detail by Schumaker and Adams (1968), allows direct determination of the difference in sedimentation coefficients of two macromolecules. We performed paired experiments using two cells, one with a 1° positive wedge window, one with a 1° negative wedge window, in the same rotor. IgG was always used as a reference solution in the 1° positive cell. All runs were performed at 20.0°. As soon as readable peaks developed, ten schlieren photographs were taken at 8-min intervals. The data were then processed to automatically compensate for the change in concentration dependence with radial dilution and were plotted to yield directly the change in sedimentation coefficient.

In the experiments where hapten is added to the antibody solution, we adjusted the concentration of hapten so that 10 μl added to 1.0 ml of antibody solution would result in the desired concentration of hapten. A second differential sedimentation analysis was always performed for each hapten by adding the same amount of hapten to IgG as was added to the DNP antibody. In this way we can compensate for any change in viscosity and density caused by the hapten but all such changes turned out to be negligible. All our values have been adjusted to the viscosity and density of water at 20°.

Sephadex G-200 Chromatography. It has been suggested (Hsia and Piette, 1969) that Sephadex G-200 chromatography can be used to separate the monomer–dimer mixture which is obtained when DNP antibody is interacted with divalent hapten (see below). A 1.5-ml sample containing 30 mg of DNP antibody plus one times α,ε-DNP-lysine (equimolar) was put onto a Sephadex G-200 column (1 × 45 cm) which had been equilibrated with 0.15 M NaCl (pH 7.0). The column was eluted with this same buffer at 3–4 ml/hr and fractions were collected every 20 min.

Sedimentation Equilibrium. Meniscus depletion sedimentation equilibrium (Wales *et al.*, 1951; Yphantis, 1964) was performed on the separated dimer eluted from the G-200 column. An apparent molecular weight was calculated for the dimer in the usual manner.

Results

Purity. The initial protein precipitation with DNP-HSA showed a classical precipitation curve. The extinction co-

TABLE II: Effect of Monovalent DNP Hapten on DNP Antibody.

Run	Description	Concn[a] (mg/ml)	$s^0_{t,s}$ (S)	$s^0_{20,w}$ (S)	$\delta s^0_{t,s}$ (S)
817	IgG	3.58	6.484 ± 0.015	6.632	−0.018 ± 0.009
817	DNP antibody plus 2× DNP-glycine	2.70	6.466 ± 0.012	6.613	
818	IgG	3.58	6.368 ± 0.007	6.513	−0.027 ± 0.007
818	DNP antibody plus 4× DNP-glycine	2.70	6.341 ± 0.010		
819	IgG	3.58	6.434 ± 0.010	6.581	−0.001 ± 0.010
819	IgG plus 2× DNP-glycine	3.58	6.434 ± 0.014		
847	IgG	4.71	6.485 ± 0.010	6.633	−0.017 ± 0.009
847	DNP antibody plus 2× ε-DNP-lysine	4.87	6.468 ± 0.008	6.615	
848	IgG	4.71	6.502 ± 0.011	6.650	0.000 ± 0.007
848	DNP antibody plus 4× ε-DNP-lysine	4.87	6.502 ± 0.012		
849	IgG	4.71	6.537 ± 0.005	6.686	+0.011 ± 0.007
849	IgG plus 2× ε-DNP-lysine	4.71	6.549 ± 0.007		

[a] The concentration is determined either by the optical density at 280 mμ, by weighing the area under the schlieren peak, or by planimetry of the area under the schlieren peak.

efficient of the DNP-HSA in 0.1 M NaOH was found to be 3.08 ml/mg at 290 mμ and 11.7 ml/mg at 360 mμ. The precipitate from the initial precipitation dissolved completely to form a clear solution upon interaction with 0.1 M DNP-glycine.

The final yield of purified antibody was calculated (Eisen *et al.*, 1954) to be 47%. The optical density at 360 mμ of the redissolved lyophilized preparation allowed us to calculate that 9% of all available binding sites (assuming two binding sites per antibody molecule) had residual DNP-glycine bound. The schlieren pattern was symmetrical, unlike the IgG samples which always had a small leading shoulder.

Extinction Coefficient. The extinction coefficient of the purified DNP antibody was found to be 1.57 ml/mg at 279 mμ (the wavelength of maximum absorption of the purified protein preparation).

Spectral Shifts. It is known that DNP haptens undergo a bathochromic spectral shift when bound to antibody (Eisen and Siskind, 1964). We found that the two monovalent haptens, DNP-glycine and ε-DNP-lysine, showed a pronounced *decrease* in extinction coefficient along with the bathochromic shift when added to the antibody solution. The two divalent haptens, however, showed a pronounced *increase* in extinction coefficient along with the bathochromic shift (see Figures 1 and 2). In all cases, it is interesting to note that adding the haptens to the IgG control causes no spectral shift, but does seem to cause a slight increase in the extinction coefficient of each hapten.

Titration. The results of the titration of DNP antibody with DNP-glycine are shown in Figure 3. We have plotted the difference in optical density at 340 mμ for IgG and DNP antibody *vs.* the amount of hapten added. The curve shows that at least 90% of the antibody seems to bind hapten.

Differential Sedimentation. GENERAL REMARKS. All differential sedimentation runs were performed with rabbit IgG as a reference solution in the 1° positive cell and the sample in the 1° negative cell. Two independent runs show that the DNP antibody has a slightly smaller sedimentation coefficient than IgG (see Table I). The average value, $\delta s^0_{20,w} = -0.040$ S, was used in all subsequent calculations.

MONOVALENT HAPTEN. Treatment with monovalent hapten yielded no apparent change in the schlieren pattern of the DNP antibody. Table II shows the results of six paired runs with the two monovalent haptens DNP-glycine and ε-DNP-

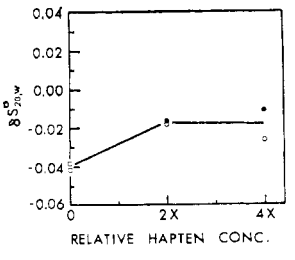

FIGURE 4: A plot of $\delta s^0_{20,w}$ *vs.* the relative concentration of monovalent hapten. Points are shown for differential sedimentation runs (see Tables I and II) on anti-DNP antibody with no hapten present (□), with DNP-glycine present (○), and with ε-DNP-lysine present (●).

TABLE III: Per Cent Change in Frictional Ratio of DNP Antibody upon Interaction with Monovalent DNP Hapten.

Unbound DNP Antibody Compared with	% Change in Frictional Ratio[a]
DNP antibody plus 2× DNP-glycine	−0.04
DNP antibody plus 4× DNP-glycine	+0.08
DNP antibody plus 2× ε-DNP-lysine	+0.03
DNP antibody plus 4× ε-DNP-lysine	−0.06

[a] A positive sign indicates an increase in frictional coefficient, while a negative sign indicates a decrease in frictional coefficient.

TABLE IV: Effect of Divalent DNP Hapten on Monomeric DNP Antibody.

Run	Description	Concn[a] (mg/ml)	$s^0_{l,s}$ (S)	$s^0_{20,w}$ (S)	$\delta s^0_{l,s}$ (S)
838	IgG	3.58	6.398 ± 0.005	6.544	+0.006 ± 0.007
838	DNP antibody plus 0.5× α,ε-DNP-lysine	3.34	6.405 ± 0.006	6.551	
834	IgG	3.69	6.397 ± 0.009	6.543	+0.020 ± 0.010
834	DNP antibody plus 1× α,ε-DNP-lysine	2.60	6.417 ± 0.007	6.563	
837	IgG	3.58	6.441 ± 0.009	6.588	+0.065 ± 0.007
837	DNP antibody plus 2× α,ε-DNP-lysine	3.51	6.506 ± 0.010		
840	IgG	3.58	6.446 ± 0.008	6.593	−0.001 ± 0.008
840	IgG plus 2× α,ε-DNP-lysine	3.58	6.445 ± 0.006		
843	IgG	4.71	6.468 ± 0.008	6.616	+0.008 ± 0.011
843	DNP antibody plus 0.5× di-DNP-cystine	3.94	6.476 ± 0.007	6.624	
841	IgG	4.71	6.541 ± 0.009	6.690	+0.027 ± 0.008
841	DNP antibody plus 1× di-DNP-cystine	3.50	6.568 ± 0.009	6.718	
844	IgG	4.71	6.341 ± 0.010	6.486	+0.016 ± 0.012
844	DNP antibody plus 1× di-DNP-cystine	3.11	6.357 ± 0.006	6.502	
842	IgG	4.71	6.419 ± 0.010	6.565	+0.063 ± 0.010
842	DNP antibody plus 2× di-DNP-cystine	3.36	6.482 ± 0.007		
850	IgG	4.71	6.497 ± 0.010	6.645	−0.007 ± 0.008
850	IgG plus 2× di-DNP-cystine	4.71	6.490 ± 0.007		
894	IgG	4.71	6.533 ± 0.011	6.682	+0.059 + 0.012
894	DNP antibody plus 4× di-DNP-cystine	5.77	6.591 ± 0.014		
895	IgG	4.71	6.457 ± 0.004	6.604	+0.049 ± 0.007
895	DNP antibody plus 4× di-DNP-cystine	2.63	6.506 ± 0.006		

[a] This is the concentration for monomer alone when monomer and dimer are present simultaneously, determined from the area under the schlieren peak.

lysine. 2× hapten means that the molarity of monovalent hapten was twice that of antibody, *i.e.*, the number of available antibody sites and the number of hapten molecules in the solution were equivalent.

The changes in sedimentation coefficient are corrected, from the control runs, for any changes in viscosity and density due to excess hapten. This gives $\delta s^0_{20,w}$ directly. The values for $\delta s^0_{20,w}$ *vs.* relative hapten concentration are plotted in Figure 4. It is seen that the sedimentation coefficient increases slightly upon hapten binding. But, as has been pointed out (Schumaker, 1968), the hapten binding causes an increase in molecular weight which itself would cause a slight increase in the sedimentation coefficient of the antibody molecule. For this reason, we calculate the per cent change in the frictional ratio of the macromolecule, taking the increase in molecular weight, due to hapten binding, into account. Basically, we calculate $\delta q/q$

$$q = \frac{f_{he}}{f_{as}} = \frac{\text{frictional coefficient of hydrodynamic ellipse}}{\text{frictional coefficient of anhydrous sphere}} \quad (1)$$

and

$$\delta q = q' - q \quad (2)$$

where the primed quantity denotes antibody after the addition of hapten and the unprimed quantity denotes antibody before the addition of hapten. $\delta q/q$ is therefore the per cent change in frictional ratio of the antibody molecule subsequent to hapten binding. The results of the calculations are shown in Table III.

DIVALENT HAPTEN. Treatment of DNP antibody with divalent hapten results in the formation of monomer and

TABLE V: Per Cent Change in Frictional Ratio of Monomeric DNP Antibody upon Interaction with Divalent DNP Hapten.

Unbound DNP Antibody Compared with	% Change in Frictional Ratio
DNP antibody plus 0.5× α,ε-DNP-lysine	−0.41
DNP antibody plus 1× α,ε-DNP-lysine	−0.62
DNP antibody plus 2× α,ε-DNP-lysine	−1.00
DNP antibody plus 0.5× di-DNP-cystine	−0.38
DNP antibody plus 1× di-DNP-cystine	−0.59
DNP antibody plus 2× di-DNP-cystine	−0.91
DNP antibody plus 4× di-DNP-cystine	−0.86

TABLE VI: Formation of Dimeric DNP Antibody upon Interaction with Divalent DNP Hapten.

Run	Description	Concn[a] (mg/ml)	$s^0_{t,s}$ (S)	$s^0_{20,w}$ (S)	$\delta s^0_{t,s}$[b] (S)
838	DNP antibody plus 0.5× α,ε-DNP-lysine	1.44	9.240 ● 0.029	9.451	+2.834 ± 0.027
834	DNP antibody plus 1× α,ε-DNP-lysine	1.67	9.225 ± 0.014	9.435	+2.831 ± 0.017
837	DNP antibody plus 2× α,ε-DNP-lysine	0.76	9.016 ± 0.191[c]	9.222	+2.661 ± 0.208[c]
843	DNP antibody plus 0.5× di-DNP-cystine	1.10	9.451 ± 0.050	9.667	+2.975 ± 0.062
841	DNP antibody plus 1× di-DNP-cystine	1.64	9.414 ± 0.018	9.629	+2.873 ± 0.019
842	DNP antibody plus 2× di-DNP-cystine	2.24	9.378 ± 0.045	9.592	+2.955 ± 0.070

[a] This is the concentration for dimer alone when both monomer and dimer are present simultaneously, determined from the area under the schlieren peak. [b] See Table II for IgG values to which these are compared. [c] Large error is due to the low concentration which resulted in only four readable peaks.

dimer exclusively (see Figure 5). No trimer, other higher species or precipitate are formed. For the divalent haptens, 1× hapten denotes equivalent molarity of hapten and antibody. (Thus, in terms of reactive groups, 1× divalent hapten corresponds to 2× monovalent hapten.) Table IV shows the results of paired runs in which the sedimentation coefficients of the monomeric species were calculated. Figure 6 is a plot of $\delta s^0_{20,w}$ *vs.* relative hapten concentration, calculated in an analogous manner to that described for monovalent hapten. The calculation of the per cent change in frictional ratio, as previously described, gives the values shown in Table V.

The sedimentation coefficients for the dimeric species were also calculated and are presented in Table VI.

Sephadex G-200 Chromatography. Figure 7 shows the schlieren patterns of the sample before it was applied to the column and after it was eluted. Samples of the eluent for the sedimentation velocity studies were taken from the apices of both the monomer and dimer peaks. It is seen that the separation is not complete, but monomer- and dimer-enriched peaks are obtained.

Sedimentation Equilibrium. The sample of dimer used in the sedimentation equilibrium study was obtained from the leading fraction of the dimer peak. This should contain almost pure dimer. The concentration was 0.70 mg/ml. A plot of log $[Y(r) - Y_0]$ *vs.* r^2 is shown in Figure 8. The calculations give an apparent molecular weight of $2.75 \times 10^5 \pm 0.02 \times 10^5$.

Discussion

The present study seems to show that interaction of anti-DNP antibody with monovalent hapten causes no conformational change in the antibody molecule as detected by differential sedimentation, while interaction with divalent hapten causes a slight (about 1%) change in frictional ratio of the

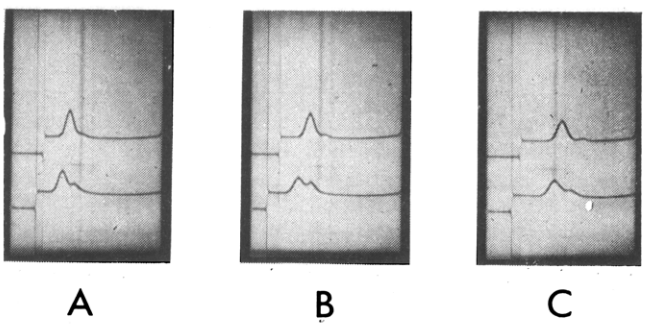

FIGURE 5: Schlieren photographs of monomer–dimer mixtures from interaction of anti-DNP antibody with 0.5 × di-DNP-cystine (A), 1 × di-DNP-cystine (B), and 2 × di-DNP-cystine (C). The top pattern in each photograph is the IgG reference solution while the bottom pattern is the anti-DNP antibody. A small leading shoulder is present in all the IgG control peaks.

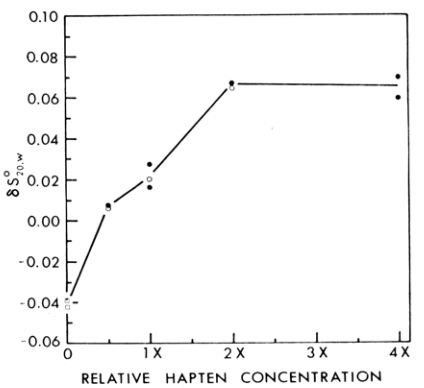

FIGURE 6: A plot of $\delta s^0_{20,w}$ of the monomeric species *vs.* the relative concentration of divalent hapten. Points are shown for differential sedimentation runs (see Tables I and IV) on anti-DNP antibody with no hapten present (□), with α,ε-DNP-lysine present (○), and with di-DNP-cystine present (●).

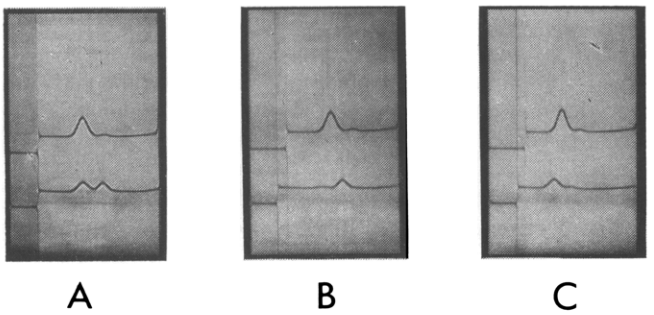

FIGURE 7: Schlieren photographs of eluents from Sephadex G-200 chromatography. Part A is the DNP-antibody after interaction with $1\times$ α, ϵ-DNP-lysine but before passage through the column, part B is from the apex of the dimer peak, and part C is from the apex of the monomer peak.

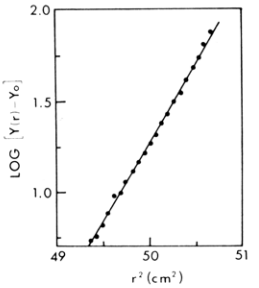

FIGURE 8: A plot of $\log [Y(r) - Y_0]$ *vs.* the radial distance squared to calculate the molecular weight of the antibody dimer isolated from the Sephadex G-200 column. The concentration of antibody was 0.7 mg/ml and the operating speed was 10,590 rpm.

antibody molecule. Upon interaction with divalent hapten, we find an increase in sedimentation coefficient of the macromolecule, which corresponds to a decrease in frictional ratio.

It is interesting to note that we found monomer and dimer exclusively upon interaction with both divalent haptens, *i.e.*, no higher order species were detectable in schlieren photographs. It has been reported (Velick *et al.*, 1960) that interaction of anti-DNP antibody with the divalent hapten, α,ϵ-DNP-lysine resulted in turbidity after the point of 1 mole of hapten/2 moles of antibody. They report formation of precipitates even in very dilute solutions of antibody (1 μN). In a more dissociable type of divalent hapten–antibody system (antiarsanilic acid antibody) no precipitate was found, but small soluble chains were detected by the light-scattering method (Epstein *et al.*, 1956). These authors also found that the weight-average degree of association of antibody molecules linked together by hapten rarely exceeds two. There was no turbidity ever detected in our preparations from 0.5 mole of hapten/mole of antibody to 4 moles of hapten/mole of antibody. Also, the amount of dimer decreased in the presence of excess divalent hapten until at 4 moles of hapten/mole of antibody, there was only a slight leading shoulder of dimer present with all the rest present as monomer. The amount of dimer also decreased when less than 1 mole of divalent hapten was present per mole of antibody with the maximum amount of dimer formed at approximately equimolar quantities of divalent hapten and antibody. This observation is in good agreement with findings for the antiarsanilic acid–antibody system (Epstein *et al.*, 1956). These qualitative observations were true for both α,ϵ-DNP-lysine and di-DNP-cystine.

In monomer–dimer mixtures formed upon interaction with divalent hapten, there are several possible species of monomer and dimer. All these possibilities are shown schematically in Figure 9. For the sake of simplicity, we will ignore the fact that 9% of the antibody had residual DNP-glycine bound and assume that all the antibody participated homogeneously in our studies.

Epstein (Epstein *et al.*, 1956) comes to several important conclusions about the thermodynamic behavior of the divalent arsanilate hapten–antibody system which can be extended to the present study: (1) the extent of association of hapten and antibody is governed by an intrinsic equilibrium constant; (2) the solution formed upon mixing antibody and hapten is a stable equilibrium, *i.e.*, the weight-average molecular weight as determined by light scattering remained unchanged after 1 week at room temperature; (3) the average standard free energy of formation of the antibody–hapten bond, $\Delta F°$, is -7.4 ± 0.2 kcal/mole; (4) bond formation causes an increase in entropy of about 22 ± 9 eu. For the ϵ-DNP-lysine–anti-DNP antibody system, a value of -6.8 kcal/mole has been found for $\Delta F°$ (Carsten and Eisen, 1955).

Using a rough value for $\Delta F°$ of -7 kcal/mole for our system, we can perhaps predict which monomeric and dimeric species are present in any mixture by comparing interconversions between species containing the same number of antibody and hapten molecules. If equimolar amounts of antibody and hapten are present, monomer form (a) would be much more favorable than form (b) since about -13.6

HAPTEN : ANTIBODY RATIO	SPECIES	MONOMERS
1:1	(a)	
1:1	(b)	
2:1	(c)	
		DIMERS
2:2	(d)	
2:2	(e)	
1:2	(f)	
3:2	(g)	

FIGURE 9: A schematic representation of the possible monomeric and dimeric species formed upon interaction of anti-DNP antibody with divalent hapten. The antibody molecule is depicted as Y shaped having three globular regions (□) connected by two somewhat flexible regions (∿). Bound hapten is shown as a solid line.

kcal/mole would be gained[2] for a as opposed to −7 kcal/mole for b. Likewise, dimeric form d would be strongly favored over e. The free energies of the interconversions between the other forms depend upon the amounts of antibody and/or hapten present. But in the concentration range of our experiments, it may be calculated that forms a and d are still the most highly favored monomeric and dimeric species. For example, even in the presence of excess hapten, monomeric species a is more favorable than c by an entropic free-energy term equal to $\Delta F = RT$ log (free hapten). However, it is possible that monomeric form a is sterically excluded due to an unfavorable location of the two antigen combining sites. In this case, the dimeric species d and f would be favored over b and c at low hapten:antibody ratios, but the monomeric form c would be favored at high hapten:antibody ratios.

The results with the G-200 column lead us to several pertinent conclusions. First, the fact that the monomer and dimer can be separated on Sephadex and in the ultracentrifuge either means that the equilibrium between the two forms is very slow or else there are two different species of molecule present: those which can form dimer and those which cannot. The latter seems more plausible in view of the very lengthy time which elapsed (2 months) between the G-200 chromatography and the sedimentation equilibrium studies. The fractions were stored at 4° during this time. They contain approximately 1× hapten. The dimeric species has an apparent molecular weight of $2.75 \times 10^5 \pm 0.02 \times 10^5$ which is twice the molecular weight of monomeric IgG which has a molecular weight of ~140,000 (Small and Lamm, 1966). The linearity of the sedimentation equilibrium curve (see Figure 8) also supports the contention that no detectable monomer is present. The monomer fractions, as shown by sedimentation velocity studies, also maintained their integrity after 2 months at 4°. This, too, tends to support the idea of two different types of molecules.

Secondly, we must consider the equilibrium between the antibody and hapten itself. Kinetic studies (Day *et al.*, 1963) show that the rate of association of ε-DNP-lysine and antibody is 5.0×10^7 (M sec)$^{-1}$ and the rate of dissociation is 1.1 (sec^{-1}) at 4°. This would allow us to predict that the purified dimer should form monomer upon interaction with excess divalent hapten. Indeed, if we perform such an experiment, the dimeric form goes almost completely to monomer when 4 moles of divalent hapten/mole of antibody is added. Experiments are now in progress to further test this reversibility.

Finally, we come to the question of the meaning of the slight increase in sedimentation coefficient of the monomeric species upon interaction with divalent hapten. Since there is no such change upon interaction with monovalent hapten, we can probably rule out the actual formation of the active site. This would be in good agreement with the kinetic studies of antihapten systems (Day *et al.*, 1962, 1963; Froese *et al.*, 1962; Froese and Sehon, 1965; Froese, 1968) which indicate that few if any conformational changes occur prior to or during the process of combination of antibody and hapten.

There are several things which one can think of which would give an apparent increase in sedimentation coefficient of the monomer antibody molecule upon interaction with divalent hapten, excluding the increase in molecular weight which was already accounted for. The first possibility is that the molecule spends part of its time as dimer. This seems ruled out since the monomer and dimer separate in the centrifuge and on the G-200 column with the isolated peaks remaining stable up to 2 months. Also, the greatest observed change in sedimentation coefficient occurs when the least amount of dimer is present (4 moles of hapten/mole of antibody). The next two possibilities depend on whether we have monomeric forms a or c present. If c were present, we can only surmise that some sort of interactions, possible only with the large divalent hapten and not the smaller monovalent hapten, occur. Perhaps there is some sort of hydrophobic interaction which makes the molecule more compact, thereby increasing its sedimentation rate. If a were present, this would be a more compact configuration than the original Y-shaped molecule and might be expected to give an increase in sedimentation coefficient and decrease in frictional coefficient (C. Warner, unpublished calculations). Also, the presence of this species is very attractive from the standpoint of the G-200 results. This monomeric form would not be able to form dimer since the molecule is internally cross-linked. Thus, we could account for the fact that the monomer and dimer are separable. It will be interesting to develop techniques to test whether monomeric species a or c or both are present in any given solution.

[2] A small correction to $\Delta F°$ results from the consideration of the four equivalent species of type b and two of type a.

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Attachment of Reticulocyte Ribosomes to Erythroid Cell Membranes *in Vitro**

Edward R. Burka† and Leonard F. Schickling

ABSTRACT: A proportion of the total ribosomes in the erythroid cell is closely bound to the cell membrane. An *in vitro* system is described in which free reticulocyte polyribosomes and monoribosomes become associated with reticulocyte cell membranes. Binding occurs at 0 and 37° and the number of receptor sites on the cell membrane appears to be limited. The number of these sites, which are not destroyed by exposure to trypsin, declines with increasing cell maturity. Free ribosomes which become associated with the cell membrane assume the sedimentation characteristics and chemical reactivity of authentic membrane-bound reticulocyte ribosomes, indicating that binding to structural components of the cell membrane is responsible for the alteration of ribosome properties. The amount of nonribosomal protein associated with membrane-bound ribosomes appears to influence their resistance to breakdown upon exposure to ethylenediaminetetraacetic acid.

Approximately 18% of ribosomes in reticulocytes of all degrees of maturity is closely bound to the cell membrane (Schreml and Burka, 1968; Burka, 1968). The significance of the binding, as well as the exact means of attachment, remains unknown (Schreml and Burka, 1968; Hendler, 1965). Both pulse-labeling experiments (Burka *et al.*, 1967) and exchange studies in an *in vitro* system (Burka, 1969) indicate that these membrane-bound erythroid cell ribosomes freely exchange with the pool of free ribosomes. The present report describes an *in vitro* system in which free erythroid cell ribosomes became attached to reticulocyte membranes, providing an opportunity to investigate the biological function and properties of membrane-bound ribosomes. The binding sites for ribosomes on the cell membrane appear to be limited in number and decrease as the cell matures. In becoming bound to the cell membrane, free ribosomes assume the physicochemical characteristics of authentic membrane-bound reticulocyte ribosomes, indicating that environmental factors related to the attachment of ribosomes are able to influence their reactivity.

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Materials and Methods

The analytical and preparative methods used have been described in detail in previous communications (Schreml and Burka, 1968; Burka, 1968, 1969; Burka *et al.*, 1967).

Isolation of Cell Components and Gradient Centrifugation. Ribosomes and erythroid cell components were isolated from the blood of either normal rabbits or rabbits with a phenylhydrazine-induced reticulocytosis. Washed packed cells were lysed by addition of four volumes of a solution of 1.5×10^{-3} M $MgCl_2$ in 1×10^{-3} M Tris (pH 7.4) (solution A). Free and membrane-bound ribosomes were isolated from membrane-free hemolysates or washed cell membranes (Burka *et al.*, 1967) in the presence of 0.2% deoxycholate (Schreml and Burka, 1968). Sucrose density gradient centrifugation was carried out in the Spinco Model L-2 preparative ultracentrifuge, using either the SW-41 or SW-25 rotor, under the conditions described in the legends to the figures. The location of [^{32}P]ribosomes in the gradient was determined as previously described (Schreml and Burka, 1968).

Attachment of Free Ribosomes to Cell Membranes. The method for attaching free reticulocyte ribosomes to washed cell membranes is shown schematically in Figure 1. Free ribosomes (6–13 mg), labeled with ^{32}P *in vivo* (Burka *et al.*, 1967), were incubated at 37° for 45 min with 4–8 ml of a suspension of washed cell membranes, containing between 8 and 10 mg of protein per ml, in solution A. The concentration of ribosomes in the incubation medium approximated their concentration in intact reticulocytes (Burka, 1968). The entire incubation mix-