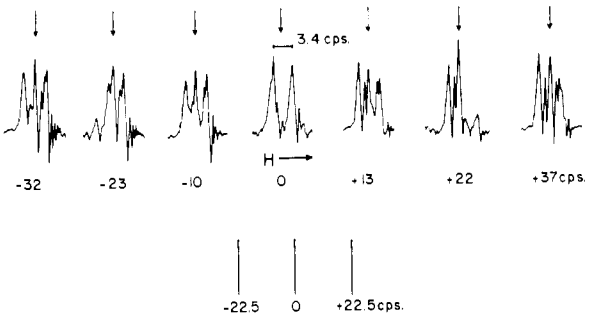

Fig. 1.—Top: spectra of the C^{13} satellite of the methylene protons (60 Mc./sec.) of CH_2DOH under conditions of rapid exchange of the hydroxyl protons and with double-irradiation at about 15.1 Mc./sec. The frequency of the second radio-frequency field relative to the resonance frequency of the center C^{13} resonance is shown under each spectrum. The arrows correspond to the center of the satellite in the unperturbed spectrum. Bottom: diagrammatic representation of the center triplet of the C^{13} spectrum of CH_2DOH at constant field.

to determine the relative signs of $J_{C^{13}H}$ and J_{gem-HH} (or J_{vic-HH}). This,[4] in effect, would allow an assignment of absolute signs to the experimentally determined J_{vic}, J_{gem} and other proton–proton coupling constants.

By using a modified spin-decoupling technique, we have now found that $J_{C^{13}H}$ and J_{gem-HD} (and hence J_{gem-HH}) are of opposite signs in CH_2DOH. If the proton spectrum is to be observed, the double-irradiation method[5] for the determination of relative signs would involve, in the present case (or in simpler systems, *e.g.*, RRC^{13} DH), "decoupling" the C^{13} for a particular deuterium spin state. This is not possible, because of the relative magnitudes of the coupling constants (*e.g.*, $J_{C^{13}-H}$,140.6; $J_{C^{13}-D}$, 22.5; J_{HD}, 1.7 cps. for CH_2DOH). However, the desired result can be achieved if the C^{13} is merely perturbed for a particular deuterium spin state, but not for others. This can be done by irradiating the C^{13} with a relatively weak magnetic field H'' ($\gamma_{C^{13}}H''/2\pi << J_{C^{13}-D}$) at a frequency corresponding to a single C^{13} transition. The effect on the proton spectrum then can be calculated from the double-irradiation theory of Bloom and Shoolery.[6]

If the proton spectrum is observed by a frequency-sweep method, the affected lines (*e.g.*, the C^{13} satellites of $CHCl_3$) are symmetrically split by about $\gamma_{C^{13}}H''/2\pi$, when the frequency of the C^{13} decoupling field is equal to either of the two C^{13} transitions. Alternatively, if the proton spectrum is observed by a field-sweep method, and the frequency of the C^{13} field is equal to either of the two C^{13} transitions when the magnetic field corresponds to the proton resonance of one of the C^{13} satellites, then that C^{13} satellite will be split[7] into a doublet. The other C^{13} satellite will not be affected if $\gamma_{C^{13}}H''/2\pi << J_{C^{13}-H}\,\gamma C^{13}/\gamma_H$.

(4) Dr. Martin Karplus (private communication) has reached similar conclusions and has suggested experiments making use of the normal[5] double-irradiation method for relative sign determination.

(5) D. F. Evans and J. P. Maher, *Proc. Chem. Soc.*, 208 (1961).

(6) A. L. Bloom and J. Shoolery, *Phys. Rev.*, **97**, 1261 (1955). See also R. Freeman and D. H. Whiffen, *Proc. Phys. Soc.*, **79**, 794 (1962); J. D. Baldeschwieler, *J. Chem. Phys.*, **36**, 152 (1962).

(7) This has been confirmed experimentally.

In the double-irradiation experiments on CH_2DOH the C^{13} perturbing field was such that $\gamma_{C^{13}}H''/2\pi$ = 3–4 cps., and the high-field C^{13} satellite of the methylene protons was observed by the usual field-sweep method with the magnetic field increasing from left to right. The extent of the field-sweep (about 4 cps.) in observing the spectrum corresponds to a shift of the C^{13} resonance of only 1 cps., and thus does not lead to complications. Because the frequency of the perturbing field is constant to only ± 1 cps. in the present experiments, the lines of the doublet are broadened and have much less than their correct heights. It is, however, very easy to see which line of the original triplet is being perturbed (*i.e.*, split into a doublet), as this line disappears from the spectrum. The nine frequencies of the C^{13} resonance were located by such experiments and showed the correct spacings. Figure 1 shows the effect when the frequency of the decoupling field is in the neighborhood of the center triplet of the C^{13} resonance. The three lines of the proton spectrum, and likewise of the C^{13} spectrum, arise from the three possible (+1, 0, −1) deuterium spin states. When the frequency corresponds to the low-, middle- or high-frequency lines of the C^{13} triplet, it is the low-, middle-, or high-field lines, respectively, of the proton spectrum which are perturbed. Frequencies which are different from these by more than 10 cps. are without appreciable effect. Since a low-field line in a sweep-field spectrum corresponds to a high-frequency line in a frequency-sweep spectrum, and *vice versa*, the coupling constants $J_{C^{13}-H}$ and J_{gem-HD} are of opposite signs. Since the magnetic moments of H and D have the same signs, $J_{C^{13}-H}$ and J_{gem-HH} are also of opposite signs. If the positive sign of $J_{C^{13}-H}$ is accepted, then J_{gem-HH} is negative in methanol, and probably in general, provided the carbon atom concerned is sp^3 hybridized. Thus, the calculated sign of the geminal coupling constant is in disagreement with experiment.

The spectra were taken on a Varian V-4302 spectrometer equipped with a "spin-decoupler" made by NMR Specialties. I wish to thank Dr. L. Leitch for a sample of deuteriated methanol.

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RECEIVED JULY 12, 1962

THE DIRECT MEASUREMENT OF THE RATE OF A HAPTEN–ANTIBODY REACTION

Sir:

We wish to report the direct measurement of the rate of the bimolecular reaction of a hapten (Hp) and its specific antibody (Ab). Such reactions generally are so rapid that in an earlier attempt to measure the rate by a spectrophotometric stopped-flow method, we found that half or more of the reaction was completed by the time measurements could be started.[1] Recourse recently has been had to equilibrium perturbation methods of measurement,[2] and while such methods

(1) J. M. Sturtevant, L. Wofsy and S. J. Singer, *Science*, **134**, 1434 (1961).

(2) A. Froese, M. Eigen and A. Sehon, *Can. J. Chem.*, in press.

are versatile, they measure forward rates only indirectly, and this may be a disadvantage in a system which is kinetically heterogeneous.

Velick, *et al.*,[3] have found that the reaction of antibodies with specific haptens having suitable optical absorption properties results in a substantial quenching of the tryptophan fluorescence of the Ab molecule. By means of a fluorometric stopped-flow apparatus[4] capable of following reactions with a half-time of a few milliseconds, we have utilized this highly sensitive effect to measure directly the rate of reaction of Ab specific for the 2,4-dinitrophenyl (DNP) group with the Hp's ε-N-DNP-L-lysine and N-DNP-ε-aminocaproic acid.[3]

The purified anti-DNP Ab[5] was obtained from a single bleeding from one rabbit.[6] It never was subjected to lyophilization, but was stored at 4° as a precipitate under 50% saturated $(NH_4)_2SO_4$ until needed. It was then dialyzed thoroughly into a phosphate–NaCl buffer at pH 7.4,[3] and the protein concentration determined from $\epsilon^{1\%}_{1cm} = 14.6$ at 279 mμ. The molecular weight of Ab was taken as 1.6×10^5. Equilibrium titrations in the fluorometric apparatus[3] with ε-N-DNP-L-lysine (m.p. 177–178) and N-DNP-ε-aminocaproic acid (m.p. 131–134°) yielded the value 1.5 for the average number of binding sites per protein molecule in the Ab solutions, and gave intrinsic equilibrium dissociation constants, K, of 2×10^{-8} M and 1×10^{-8} M for the two Hp's, respectively. Rate measurements were carried out in the phosphate–NaCl buffer at 25.0° at a constant initial concentration of Ab sites of 4.7×10^{-7} M and varying concentrations of the two Hp's, with the incident and emergent monochromators of the stopped-flow instrument set at 280 and 340 mμ, respectively. With the assumption that all Ab active sites are equivalent and independent, the results were analyzed with the aid of the expression[7]

$$k_1 t = \frac{1}{\sqrt{-q}} \ln \frac{1 - x\,\dfrac{a_0 + b_0 + K - \sqrt{-q}}{a_0 + b_0 + K + \sqrt{-q}}}{1 - x} = F(t) \quad (1)$$

where $x = 0$ at $t = 0$ and $x = 1$ at equilibrium ($t = \infty$); a_0 and b_0 are the initial concentrations in moles/liter of the reactants, with $b_0 > a_0$; $-q = 4Ka_0 + (b_0 - a_0 + K)^2$; and k_1 is the second-order specific rate constant for the forward reaction.

The results of one representative series of experiments are shown in Fig. 1, plotted according to equation (1). Each curve is drawn through the points of three individual experiments with the same Ab and Hp solutions. The downward curvature exhibited in Fig. 1 is reproducible and real, and is independent of any possible uncertainty in the values of K used in equation (1). The curvature appears to reflect a decrease in the average value of k_1 as the fraction of Ab sites reacted increases. This may be seen in Table I from experiments with different ratios of Hp to Ab. This variation in k_1 might be due to an interdependence of the two sites on a given Ab molecule, and comparable experiments with univalent Ab fragments[8] are planned to test this possibility. More likely, however, the variation in k_1 reflects a heterogeneity of Ab sites. Such evidence for Ab heterogeneity is particularly significant in this case, in view of these considerations: (a) the unusually high degree of uniformity of the antigenic determinant used to elicit anti-DNP Ab[3] (Ab heterogeneity in many other cases, especially where the antigen is prepared by diazonium coupling reactions,[9,10] may result primarily from the heterogeneity of the antigen); (b) the use of a single rabbit antiserum, rather than a pool; (c) the relatively mild treatment to which the Ab was subjected; and (d) the similarity of the results with the zwitterionic Hp ε-N-DNP-L-lysine and the anionic Hp N-DNP-ε-aminocaproic acid (which suggests that the gross heterogeneity

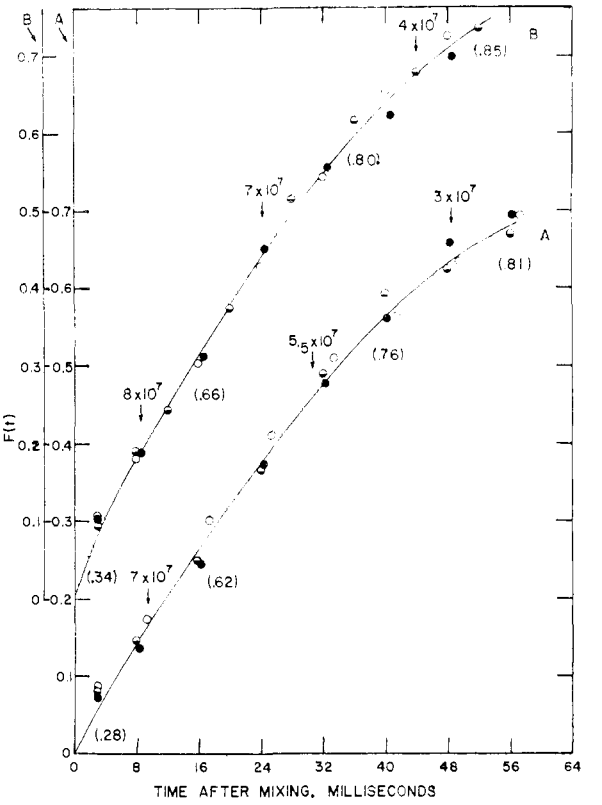

Fig. 1.—Kinetics of reaction of anti-DNP Ab with ε-N-DNP-L-lysine (A) and N-DNP-ε-aminocaproate (B) at 25.0° plotted as a second order forward, first order reverse reaction (equation (1)). Initial concentration of Ab sites, 4.7×10^{-7} M; of hapten, 9.4×10^{-7} M. Closed, open and shaded circles refer to three separate runs. Numbers in parentheses indicate fraction of total Ab sites reacted at different points on the curves. Included above the curves are apparent second order rate constants calculated from tangents at various points indicated by the arrows.

(3) S. F. Velick, C. W. Parker and H. N. Eisen, *Proc. Nat. Acad. Sci. U. S.*, **46**, 1470 (1960).

(4) J. M. Sturtevant, unpublished work.

(5) F. S. Farah, M. Kern and H. N. Eisen, *J. Exp. Med.*, **112**, 1195 (1960).

(6) We are indebted to Dr. Henry Metzger for the purification of the anti-DNP Ab.

(7) J. W. Baker and M. L. Hemming, *J. Chem. Soc.* (London), **144**, 191 (1942).

(8) R. R. Porter, *Biochem. J.*, **73**, 119 (1959).

(9) E. W. Gelewitz, W. L. Riedeman and I. M. Klotz, *Arch. Biochem. Biophys.*, **53**, 411 (1954).

(10) M. Tabachnick and H. Sobotka, *J. Biol. Chem.*, **235**, 1051 (1960).

TABLE I

APPARENT SECOND ORDER RATE CONSTANTS AS A FUNCTION OF EXTENT OF REACTION

In units of M^{-1} sec.$^{-1} \times 10^{-7}$, under conditions given in Text.

	ε-N-DNP-L-Lysine			N-DNP-ε-Aminocaproate		
Initial ratio [Hp]/[Ab sites] →	0.5	2.0	8.4	0.5	2.0	8.1
Fraction of total Ab sites reacted						
0.1	9.0	..	..	13.2	..	..
.2	7.9	..	..	11.1	..	..
.4	5.8	6.8	..	9.9	9.0	..
.6	..	6.2	3.9	..	7.7	5.9
.8	..	2.5	2.0	..	5.1	2.1
.9	..	..	1.4	..	..	1.5

in net electric charge of pure anti-DNP Ab[3] is not primarily responsible for the apparent kinetic heterogeneity).

The maximum value of k_1 for both haptens appears to be about 1×10^8 M^{-1} sec.$^{-1}$, which makes this one of the most rapid bimolecular reactions in homogeneous solution known to biochemistry. It is interesting that the same k_1 value was estimated[1] from stopped-flow experiments with anti-DNP Ab and an entirely different DNP-hapten, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonic acid, by an independent spectrophotometric technique. One would predict[11,12] for the present system that diffusion would limit the rate constant to a value of about 10^9 M^{-1} sec.$^{-1}$. Such a large value for k_1 suggests that no substantial conformational rearrangement (requiring an appreciable free energy of activation) occurs within the Ab molecule, or active site, upon specific binding of the hapten.

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(11) J. H. Wang, *J. Phys. Chem.*, **59**, 1115 (1955).
(12) G. V. Schulz, *Z. physik. Chem.*, **8**, 284 (1956).

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CONFORMATIONAL ASPECTS OF SYNTHETIC POLYPEPTIDES. VI. HYPOCHROMIC SPECTRAL STUDIES OF OLIGO-γ-METHYL-L-GLUTAMATE PEPTIDES[1]

Sir:

Absorption spectra of high molecular weight polypeptides and proteins in the far ultraviolet region have been employed for the study of their conformations in solution.[2-6] Peptides in the helical conformation have been shown to exhibit a marked decrease in intensity of the absorption band (molar extinction coefficient approximately 4,000) in the 190 mμ region compared with the same peptide in random coil conformation (molar extinction coefficient approximately 7,500). Helical structures also exhibit a new absorption band or shoulder in the 205 mμ region. Polylysine, polyglutamic acid and polyalanine are examples of synthetic polypeptides which have been studied by this technique.

We have applied the study of far ultraviolet absorption spectra to oligomeric peptides derived from γ-methyl-L-glutamate where the amino end is blocked by a benzyloxycarbonyl group.[1,7] All evidence which we have developed to date on the critical size necessary for helix formation has depended on optical activity measurements. The application of hypochromic studies from absorption spectra described in this paper provides the first data for the independent verification of the onset of helical conformations in the critical range of peptide chain length.

The solvent for our investigations was 2,2,2-trifluoroethanol which has low absorption in the 190 mμ region. A Perkin Elmer model 350 spectrophotometer was used in this work. It was kept air free by continuous flushing with dry prepurified nitrogen. This enabled us to work in the region down to 189 mμ with a slit width of less than 0.6 mm. and to 187 mμ with a slit width of less than 1.5 mm. Matched quartz cells (0.1 mm. width) were used. The concentrations of the peptides studied were in the range of 10^{-2} to 10^{-3} mole/liter.

The molar extinction coefficients (ε′) are shown in Fig. 1 as a function of wave length for various oligopeptides and for a high polymer of γ-methyl-L-glutamate. These extinction coefficients (ε′) have been corrected for the absorption of the benzyl group of the benzyloxycarbonyl blocking group in the oligopeptides.[8]

$$\epsilon'_{\text{peptide}} = \epsilon_{\text{observed}} - f_{C_6H_5CH_2} \times \epsilon_{C_6H_5CH_3}$$

where ε′ is the molar extinction coefficient per residue corrected for benzyl group absorption. $\epsilon_{\text{observed}}$ is the observed molar extinction coefficient. $f_{C_6H_5CH_2}$ is the weight fraction of the benzyl group in the molecule. $\epsilon_{C_6H_5CH_3}$ is the molar extinction coefficient of toluene in this region.

With the peptides studied the hypochromicity of the absorption band measured at 189–190 mμ commences at the nonamer, increasing in magnitude for the higher oligopeptides and the polymer. The appearance of the shoulder at the 205 mμ region also begins at the nonamer, increasing in intensity with chain length.

Assuming that the high polymer exists entirely in the helical state and the pentamer and heptamer possess no helical conformation in this solvent, we have calculated the helical content for any of the oligomers from the extinction coefficients at

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(3) P. Doty and W. B. Gratzer, *ibid.*, p. 111.
(4) K. Rosenheck and P. Doty, *Proc. Natl. Acad. Sci.*, **47**, 1775 (1961).
(5) W. B. Gratzer, G. M. Holzworth and P. Doty, *ibid.*, **47**, 1785 (1961).
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(8) J. R. Platt and H. B. Klevens, *Chem. Rev.*, **41**, 301 (1947).