References and Notes

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10. The assistance of Miss Jean M. McCarthy is gratefully acknowledged.

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Loss of Precipitating Activity of Antibody without Destruction of Binding Sites

In investigating the composition of the specific combining region of antibody, we have studied the reaction of acetic anhydride with rabbit antibody homologous to the *p*-azobenzoate group. Others have found that acetylation of various antibodies results in loss of ability to precipitate with antigen (*1*). Marrack and Orlans (*2*) reported that when rabbit antibodies against several different antigens were acetylated, they would no longer precipitate, but that a large fraction of each acetylated antibody coprecipitated when added to a mixture of antigen and untreated antibody. They concluded that the failure to precipitate was due either to electrostatic repulsion among protein molecules or to deformation of the antibody molecule resulting from the increased negative charge which accompanies acetylation of amino groups.

In the present work, acetylated preparations of rabbit antibody homologous to the *p*-azobenzoate group were tested for ability to precipitate an ovalbumin-*p*-azobenzoate test antigen, and also to bind homologous hapten [by equilibrium dialysis (*3, 4*)]. Binding experiments provide direct information bearing on the number of combining sites present. It was found that mild acetylation results in complete loss of precipitating activity but that the ability to bind homologous hapten was affected only slightly. The results confirm the hypothesis of Marrack and Orlans and show that an antibody with both combining sites intact may fail to precipitate. Another conclusion is that the nonspecific electrostatic effect in the interaction of this antibody with the negatively charged homologous hapten is small.

Experiments were carried out with antisera obtained from ten rabbits repeatedly injected with a bovine γ-globulin-*p*-azobenzoate antigen, prepared by coupling 30 mg of diazotized *p*-aminobenzoic acid to 1 g of bovine γ-globulin (*4, 5*). The γ-globulin fraction of the pooled antisera was separated by precipitation with sodium sulfate (*6*). Free electrophoresis showed the presence of only one peak, corresponding to γ-globulin. Two 5 ml portions of the globulin (32.4 mg/ml) were acetylated at 0°C by addition, with a microburette, of several portions of acetic anhydride over a period of 15 minutes. The amounts of the reagent used in each experiment are given in Table 1. The mixture was stirred continuously and allowed to react for 1 hour; *p*H was maintained at 8.0 to 8.5 with 0.1*N* NaOH. An untreated control and the acetylated samples were dialyzed overnight against 10 liters of saline-borate buffer at 3° to 5°C and adjusted to the same final protein concentration.

The results are shown in Table 1. The unacetylated globulin and sample A (about 20 percent of the amino groups acetylated) exhibited typical precipitin curves. However, the entire curve for A was displaced downward and, as is indicated in the table, only 41 percent as much precipitate formed at the optimum. No precipitation or turbidity was observed in mixtures of antigen and sample B, which was more extensively acetylated (85 percent of amino groups reacted). In contrast to these results, it is evident (Table 1) that the ability to bind the homologous hapten, *p*-iodobenzoate-I^{131} (*7*) was not greatly affected by acetylation. There was no appreciable difference in the concentration of hapten bound by the control or sample A. In the case of sample B, which formed no precipitate, the concentration of hapten bound, corrected to the same free hapten concentration (*8*), was 79 percent of that bound by the unacetylated sample. This small adverse effect of acetylation on binding may be attributed either to a decrease in the average combining constant (K), the loss of effective combining sites, or both. Some decrease in K appears likely since the hapten is negatively charged and the net negative charge per molecule in preparation B was increased by about 65 units on acetylation.

We may obtain an estimate of the maximum number of combining sites affected by assuming that K was unchanged and that the observed effects on binding were due entirely to the attack of acetic anhydride on groups in the specific combining region of the antibody. It would appear then that a maximum of about one-fifth of the sites or somewhat less than two-fifths of the molecules were affected [assuming random acetylation and the presence of two combining sites per molecule (*9*)]. It is also possible that a smaller number of sites were affected and that a decrease in K was partly or entirely responsible for the reduced binding. In any event, the data show that complete loss of precipitability may occur without a corresponding decrease in the number of sites capable of combining with hapten.

The results suggest that the failure of the acetylated antibody to precipitate is attributable to a factor other than attack of the reagent on the specific combining region. It is possible that the acetic anhydride reacts with a group in a part of the combining site which interacts with antigen but lies beyond the region making contact with *p*-iodobenzoate (*10*). However, this alternative cannot apply to the data of Marrack and Orlans (*2*), who used only protein antigens. Their explanation, that acetylated antibody does not precipitate because of electrostatic repulsion resulting from its increased negative charge, can also account for the lack of precipitation in the antiazobenzoate system (*11*). The interaction of

Table 1. Effects of acetylation on the γ-globulin fraction of rabbit anti-*p*-azobenzoate antiserum. Mean deviations given in table are based on duplicate or triplicate analyses. The procedure for acetylation is described in the text.

Sample	Ac_2O used (mg)	No. of amino groups per molecule remaining*	Antibody precipitable by antigen† (μg)	Labeled *p*-iodobenzoate bound‡ ($\times 10^6$M)
Normal globulin	0	–	–	0.21 ± 0.02
Antibody globulin (control)	0	75 ± 8§	228 ± 2	7.5 ± 0.2
Antibody globulin (A)	2.8	58 ± 1.5	93 ± 3	7.2 ± 0.8
Antibody globulin (B)	14	11 ± 1.5	0‖	6.6 ± 0.1

* From van Slyke amino nitrogen analyses. Calculation based on molecular weight 160,000.
† Precipitin reaction with 0.20 ml each of antibody and antigen. The latter was prepared by coupling 60 mg of diazotized *p*-aminobenzoic acid to 1 g of ovalbumin. Values given are for optimum antigen concentration.
‡ Protein concentration, 16.2 mg/ml. Free hapten concentration 1.71 ± 0.06, 1.75 ± 0.07 and 1.90 ± 0.17 × $10^{-6}M$, corresponding to control and samples A and B; and 2.95 ± 0.07 × $10^{-6}M$ for the normal globulin. Values for the antibody preparations are corrected for the small amount of binding by normal globulin.
§ Amino acid analysis (*12*) indicates the presence of 72 free amino groups per molecule.
‖ No turbidity or precipitation observed.

antibody with antigen should be affected more adversely by increased negative charge than that with hapten, since the protein-protein electrostatic repulsion, in the case of the reaction with antigen, is superimposed on that involving the haptenic group. Additional, intermolecular repulsions may also be involved in the formation of aggregates of antibody and antigen.

Finally, it is of interest that the nonspecific electrostatic effect in the interaction of the antibody with hapten is small. With about 65 additional negative charges in the antibody molecule, the binding of *p*-iodobenzoate by antibody was affected only to a small degree (Table 1). In untreated antibody at *p*H 8, the net negative charge per molecule is about 15 or 16 units; thus the nonspecific electrostatic free energy of combination at *p*H 8 is probably negligible. This of course does not preclude the possibility of a very large interaction of opposite charges in the specific combining region.

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References and Notes

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2. J. R. Marrack and E. S. Orlans, *Brit. J. Exptl. Pathol.* 35, 389 (1954).
3. Precipitin reactions were allowed to stand at 3° to 5°C for 5 days. Equilibrium dialysis was carried out at 5° ± 0.1°C. Except as noted, saline-borate buffer, *p*H 8.0, $\mu = 0.16$, was used. Details are given in reference *4*.
4. A. Nisonoff and D. Pressman, *J. Immunol.* 80, 417 (1958).
5. Rabbits were immunized with an antigen made by coupling 30 mg of diazotized *p*-aminobenzoic acid to 1 g of bovine γ-globulin (Armour fraction II). The coupling reaction was carried out at *p*H 9 to 9.5 at 5°C. Details are given in reference *4*.
6. R. A. Kekwick, *Biochem. J.* 34, 1248 (1940).
7. One milliliter of $10^{-6}M$ solution of *p*-iodobenzoate gave approximately 2000 count/min (about 15 times background). Counting efficiency was about 50 percent.
8. The small correction was made by use of the observation, made with a number of different preparations of this antibody (*4*), that the fraction of hapten bound, at hapten concentrations which are small compared to the antibody concentration, does not change appreciably with small changes in the free concentration of hapten.
9. For a given species of site (that is, group of sites having the same K) the concentration of hapten bound is directly proportional to the total concentration of those sites remaining, provided that K and the free hapten concentration are fixed. It follows that the concentration of hapten bound to a heterogeneous population of combining sites, under the same conditions, is proportional to the total concentration of sites. This assumes that acetic anhydride does not react selectively with sites on the basis of their combining constants.
10. Preliminary experiments indicate that the ability of the mildly acetylated antibody to bind the dye, *p*-(*p*-hydroxyphenylazo)-benzoate, is similarly unimpaired. Since this hapten is larger than *p*-iodobenzoate, this result supports the evidence against an attack on the specific combining region.
11. Dr. Schlamowitz of this laboratory, in studies on the phosphatase-rabbit antiphosphatase system, has found that acetylation of the antibody causes a delay in precipitation, but that the antibody still combines with antigen, as evidenced by coprecipitation of the complexes with horse antirabbit γ-globulin antibodies (unpublished results).
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23 January 1958

Fluorescence Activation Spectra of a Diphosphopyridine Nucleotide Dependent Dehydrogenase

In a previous communication from this laboratory (*1*), the shift and augmentation of the fluorescence spectrum of reduced diphosphopyridine nucleotide (DPNH) (*2*) in the presence of beef heart muscle lactic dehydrogenase (LDH) was reported. It was further reported that an additional shift and increase of the fluorescence spectrum occurs when L-lactate is added to the LDH-DPNH complex, presumably to form an LDH-DPNH-L-lactate complex (*3*). For DPNH, when activated by light having a wavelength of 340 mμ, maximum fluorescence emission occurs at 465 to 470 mμ. For LDH-DPNH and LDH-DPNH-L-lactate complexes, maximum emission is observed at 445 to 450 mμ and 430 to 435 mμ, respectively. Similar shifts in the fluorescence spectrum have recently been reported for other dehydrogenase systems (*4*).

Since the initial observation that the alteration of the fluorescence spectrum of DPNH in the presence of horse liver alcohol dehydrogenase is accompanied by a shift to shorter wavelengths of the *absorption* maximum of DPNH (*5*) several attempts have been made to detect a similar alteration of the absorption spectrum of DPNH in the presence of LDH. However, the magnitude of the absorption change is so small that it could be detected only with the very sensitive spectrophotometer employed by Chance and Neilands (*6*).

Since it is well known that only absorbed light can give rise to fluorescence emission, it occurred to us that in the case of LDH and DPNH, only a small fraction of the absorbed light gave rise to the fluorescence spectrum. As a result, rather pronounced changes in the fluorescence spectrum are accompanied by minute changes in the absorption spectrum. In this case, examination of the activation spectrum should reveal those changes in the absorption spectrum which give rise to fluorescence emission (*7*). Figure 1 illustrates activation spectra of DPNH, LDH, LDH-DPNH complex, and LDH-DPNH-L-lactate complex as measured in the Aminco-Bowman recording spectrophotofluorometer (*8*) in 0.2 ionic strength phosphate buffer, *p*H 6.61, at 20°C. For DPNH, LDH-DPNH complex, and LDH-DPNH-L-lactate complex, the fluorescence monochromator was set at the wavelength of maximum emission. For LDH, the fluorescence monochromator was set at 465 mμ, the wavelength of maximum emission of DPNH. The addition of pyruvate to a final concentration of $1.3 \times 10^{-4}M$ to either the LDH-DPNH or LDH-DPNH-L-lactate systems results in a rapid change to the activation spectrum of LDH alone.

It is clear from Fig. 1 that the wavelength of maximum activation of the LDH-DPNH complex is about 5 mμ less than that of DPNH, and that a further shift of 5 mμ is observed when the LDH-DPNH-L-lactate complex is formed. A striking change in the activation spectrum of LDH is seen at 285 mμ when enzyme-coenzyme and enzyme-coenzyme-L-lactate complexes are formed. Ternary complexes are also formed when structural analogs of L-lactate, such as oxalic, tartaric, tartronic, α-hydroxybutyric, malic, and ascorbic acids, are

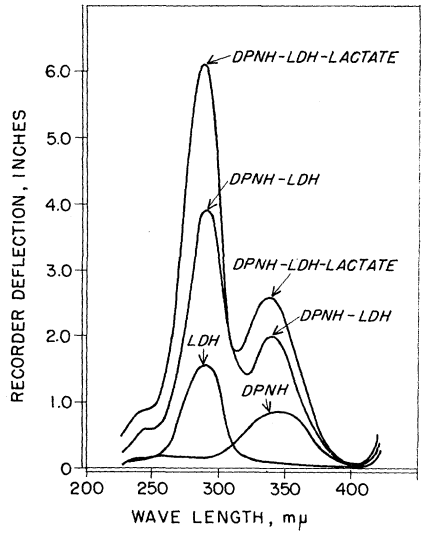

Fig. 1. Fluorescence activation spectra of lactic dehydrogenase and of lactic dehydrogenase complexes with DPNH and with DPNH and L-lactate. The intensity of fluorescence emission at a constant wavelength, as measured by recorder deflection, is plotted against the wavelength of the activating radiation. The curve labeled LDH was obtained with $9.60 \times 10^{-7}M$ LDH. The molecular weight of the enzyme was taken as 135,000 (*10*). The curve labeled DPNH was recorded at a DPNH concentration of $3.94 \times 10^{-6}M$. When DPNH and LDH were each present at the concentration used for the measurement of their separate spectra, the curve LDH-DPNH was obtained. The curve labeled LDH-DPNH-L-lactate was obtained when Na-L-lactate at a final concentration of $1.57 \times 10^{-2}M$ was added to LDH and DPNH present in the concentrations used for the other curves. The background fluorescence of phosphate buffer and L-lactate is negligible at the instrument settings employed.