BINDING OF ANTIBODIES TO NITROXIDE SPIN LABELS AND TO THE CORRESPONDING HYDROXYLAMINES

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SUMMARY: The affinities of rabbit antibodies directed against the spin-label nitroxide group have been found to be of the order of 10^6 1/mole for a number of low molecular weight water soluble haptens. It is shown that the same antibodies have almost equal binding affinities to corresponding hydroxylamines.

INTRODUCTION: The magnetic resonance spectra of biological systems (especially membranes) containing nitroxide spin labels can provide detailed structural and kinetic information concerning these systems (1). Recently it has been found possible to prepare anti-nitroxide (or anti-spin label) antibodies (2); this offers unique opportunities for the study of membrane immunochemistry, and some of these studies have already been carried out (3,4). The purpose of the present communication is to describe the binding of an anti-nitroxide IgG fraction to a number of simple water soluble nitroxide haptens. The present work also explains the otherwise unexpected result that it is in fact possible to prepare anti-nitroxide antibodies, since many nitroxide groups are often rapidly reduced to the hydroxylamine *in vivo* (1). Finally, the present work describes a related, fortuitous and

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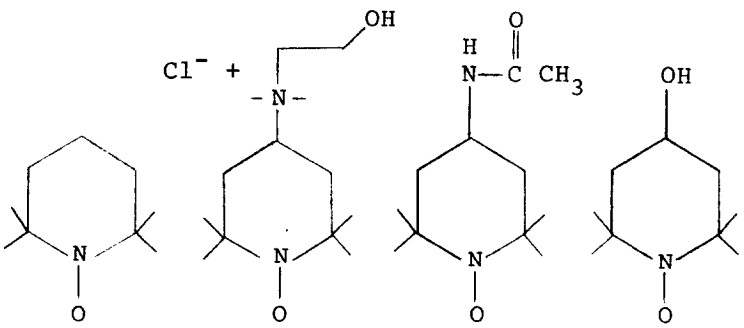
Abbreviations: PBS, phosphate-buffered saline (.01 M NaH_2PO_4; .15 M NaCl, pH = 7.3).

convenient result, namely the binding affinities of nitroxides and the corresponding hydroxylamines to antibodies are nearly equal to one another.

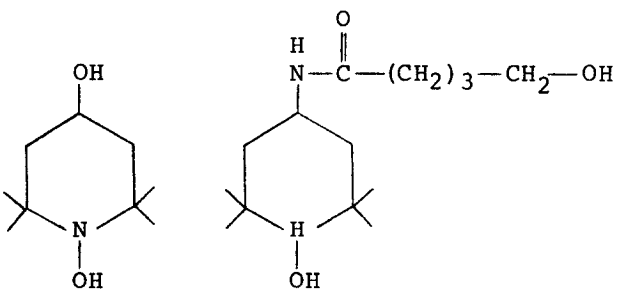
RESULTS: Antibodies were produced in hyperimmunized New Zealand white rabbits using spin-labeled hemocyanin, as described previously (2). Most of the immunoglobulins used in the present study were obtained by repeated precipitations with 30 % $(NH_4)_2SO_4$ Specific activity was shown in separate work to be exclusively IgG (2-4). For some experiments affinity purified IgG antibodies were prepared as described elsewhere (4). The nitroxide haptens



I II III IV

were prepared as described previously (1).

The hydroxylamines V and VI,



V VI

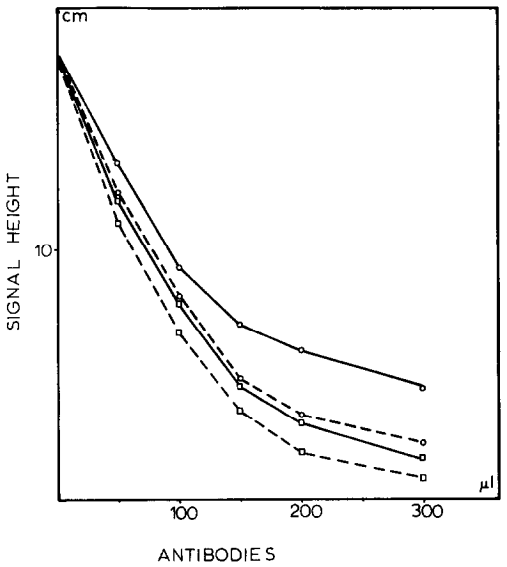


Figure 1. IgG binding to nitroxide spin labels I (O---O---), II (O—O—), III (□---□---), IV (□—□—). The "signal height" is the paramagnetic resonance derivative curve amplitude. In each case the nitroxide concentration is 3.8×10^{-6} M. Low field signal amplitudes in the absence of antibodies are equal to one another to within 10 %, but are normalized to the same amplitude in this figure. Estimated total specific IgG concentration is 5×10^{-6} M (see text); each sample was prepared using 25 μl of 5×10^{-5} M nitroxide, x μl of IgG solution, and made up to 325 μl using PBS.

were obtained by reduction of the corresponding nitroxides: the nitroxide corresponding to V was reduced with powdered zinc and acetic acid; the nitroxide corresponding to VI was reduced with 1,2 diphenylhydrazine.

Figure 1 shows the amplitude of the low-field hyperfine component (1) of haptens I-IV in aqueous PBS solutions to which have been added increasing amounts of IgG, prepared as described above.

Figure 2 shows a Scatchard plot of $10^6 r/c$, where r is the ratio of hapten bound per mole of antibody, and c is the concen-

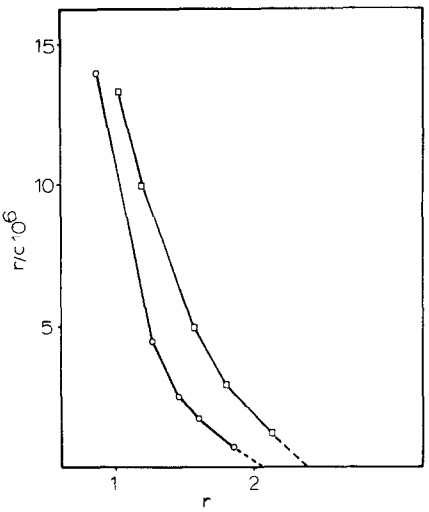


Figure 2. Scatchard plot of IgG-hapten binding, for haptens I (□) and III (○). See text for definitions of r and c.

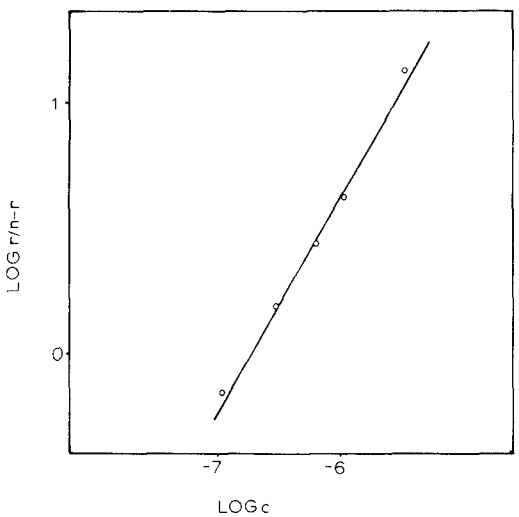


Figure 3. Sips plot for IgG binding to hapten III. See text for definitions.

tration of free hapten. The horizontal intercept is adjusted to equal two, as expected for IgG molecules with two binding sites (5). The concentrations of nitroxide-binding IgG molecules used for plots in Figure 2 were estimated crudely from an analysis of the

data on III in Figure 1, assuming a dominant population of IgG molecules with a single binding constant K. The best fit throughout the entire range was obtained with $K \simeq 5 \times 10^6$ 1/mole, and an antibody concentration of 5×10^{-6} moles/liter.

Figure 3 shows a Sips plot, corresponding to a $K = 5 \times 10^6$,

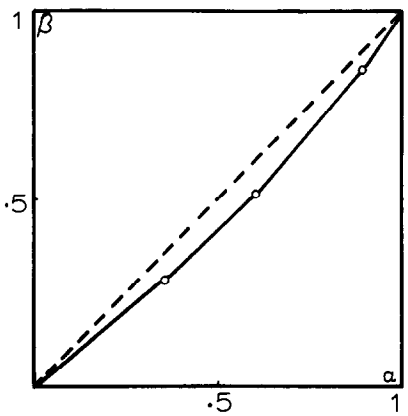


Figure 4. Competitive binding of nitroxide IV and hydroxylamine V. α is the ratio [IV]/{[IV] + [V]} where [IV] and [V] refer to total concentrations. β is the fraction of nitroxide bound to a fixed quantity of antibody as judged by the amplitude of the remaining free signal.

and an index of heterogeneity, a = 0.87, and n = 2 is the number of combining sites per molecule (5).

Figure 4 shows the competitive cross-reaction between the hydroxylamine V and the corresponding nitroxide IV. A slope of one in this plot indicates equivalent affinities for the antibodies; it will be seen that this slope is indeed near one; the hydroxylamine has a binding constant that is of the order of 10 %, higher than the nitroxide. Similar results were obtained in a comparison of III and VI.

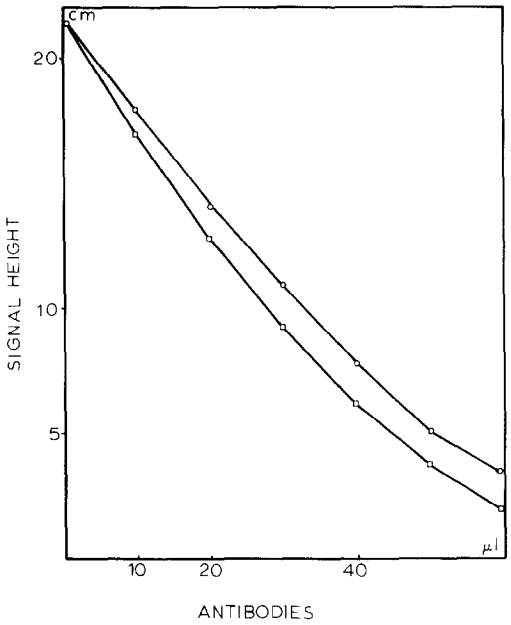


Figure 5. The binding of haptens I (O) and III (□) to affinity purified IgG. The specific IgG concentration estimated by uv absorption is 1.95 mg/ml and the hapten concentration is 0.833×10^{-5} M. Solutions were made up to a total volume of 100 μl with PBS. A rough Scatchard plot indicates that the specific IgG concentration may only be one half that estimated by uv absorption, and that the binding affinities are of the order of $1\text{-}6 \times 10^6$ l/mole.

Figure 5 shows the binding of I and III to a specific IgG fraction prepared by elution from a nitroxide-conjugated sepharose column. In this case the specific antibody concentrations were determined spectrophotometrically, and the binding constants derived for I and III from the data in Figure 5 are in the range $1\text{-}6 \times 10^6$ l/mole. Note that under these conditions the binding of haptens I and III are very nearly equal to one another.

An important feature of nitroxide spin labels has been the quantitative structural and kinetic information they provide concerning membrane and protein structure (1). One drawback to their broad applicability has been their occasional susceptibility

to chemical reduction to the hydroxylamine in some biological cells (1). The present work shows that this need not be a serious problem for a number of cellular immunochemical experiments.

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