ANTIBODIES AGAINST NITROXIDE SPIN LABELS

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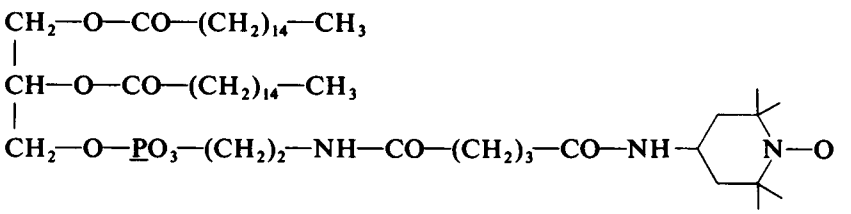
In this communication, we report preliminary studies of reactions of specific rabbit antibodies with nitroxide free radicals. Since nitroxide free radicals have been used extensively as spin labels (1), such antibodies will doubtless be useful for a variety of applications. This brief communication describes several spectral consequences of the binding of antibodies to nitroxide spin labels.

Keyhole limpet hemocyanin was alkylated with *N*-(1-oxyl-2, 2, 6, 6-tetramethyl-piperidinyl)-4-iodoacetamide (2). Rabbits were immunized by subcutaneous injection of 500 μg of the labeled protein in complete Freund's adjuvant, and then boosted by several intravenous injections of 100 μg protein in saline. Immunoglobulins were separated by repeated fractionation with $(NH_4)_2SO_4$, followed by dialysis against phosphate-buffered saline. Specific antibodies against the spin label nitroxide group were identified using paramagnetic resonance as shown here, and also using complement fixation to be described elsewhere.

When the phospholipid spin label I (preparation to be described elsewhere)



(I)

is incorporated at a concentration of 2.5 mol% in dimyristoylphosphatidylcholine vesicles, the resonance spectrum consists of three sharp resonance signals separated from one another by approximately 15 G ("weakly immobilized signal"). If this lipid mixture is sonicated so as to maximize the exposure of the nitroxide groups to the external aqueous solution, and incubated at 37° for 2 h with specific antibodies, the "strongly immobilized" resonance spectrum shown in Fig. 1 is obtained. (The relatively low amplitude weakly immobilized signal seen in Fig. 1 is due to spin labels I

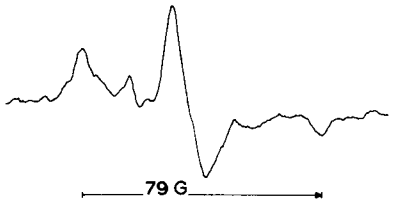


FIGURE 1

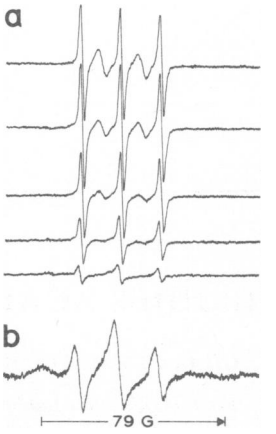


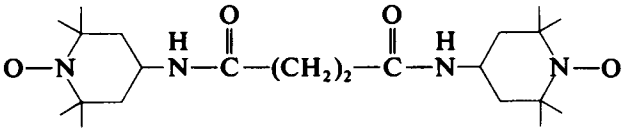
FIGURE 2

FIGURE 1 The strongly immobilized paramagnetic resonance spectrum of the lipid hapten, nitroxide spin label I (see text), at a concentration of 2.5 mol% in dimyristoylphosphatidylcholine bilayers. The nitroxide group is immobilized due to nitroxide-specific antibodies (rabbit). FIGURE 2 (*a*) The paramagnetic resonance spectra of biradical II (see text) as a function of increasing concentration of anti-nitroxide immunoglobulin (top to bottom). Phosphate-buffered physiological saline was used to prepare samples having immunogobulin concentrations approximately equal to 0/5, 1/5, 2/5, 3/5, 4/5, 5/5 of that present in the original serum, and a biradical II concentration of $\sim 5 \times 10^{-6}$ M. After 4 days at 6°, the samples were examined using a Varian E12 spectrometer and a quartz flat cell; spectra of the first five samples are shown in order of increasing immunoglobulin concentration. The sixth sample, having immunoglobulin at a level close to that in whole serum, showed no sharp component, indicating that all nitroxide groups were bound at that level. In contrast, immunoglobulin from a non-immunized rabbit had no effect on the spectrum of the biradical. A minimum specific antibody concentration of the order of 1 mg/ml is indicated for the original serum. (*b*) Same as the bottom spectrum in *a*, but distorted by using a very high modulation amplitude (4G) and power (63 mW) so as to observe the broad immobilized component.

protected from antibodies by the lipid membranes.) The restricted motion giving the strongly immobilized signal in Fig. 1 is due to binding of the nitroxide group by specific antibody. The observed separation of the outer hyperfine extrema (79 G) is, to our knowledge, the largest hyperfine separation (equal to $2T_{zz}$ in standard notation [1]) yet observed for nitroxide spin labels. This must be due to a strong specific hydrogen bond from the combining site of the antibody to the oxygen atom of the nitroxide group. This hydrogen bond leads to an increase of negative charge on oxygen, an increase in the positive charge and spin density on the nitroxide nitrogen atom, and an increase in the hyperfine splitting T_{zz}. The observed hyperfine extrema are remarkably sharp in view of the diversity of antibodies that is doubtless present.

Another consequence of nitroxide radical-antibody interaction is illustrated by the spectra in Fig. 2. These spectra show the results of increasing additions of nitroxide-

specific antibodies to a solution of the biradical II,



(II)

In the absence of antibodies, the resonance spectrum of II shows five hyperfine signals, whose relative widths are characteristic of a biradical with relatively weak spin exchange, as discussed in a review by Hudson and Luckhurst (3). The sequential changes in spectra seen in Fig. 2 are readily understood qualitatively, as follows. Low concentrations of antibodies result in antibody-bound biradicals in which one nitroxide group is held in the combining site of the antibody, giving a strongly immobilized signal, and the second nitroxide group is free, giving a sharp, three-line spectrum (weakly immobilized spectrum) with no manifestation of spin exchange interaction between the nitroxide groups. With increasing concentrations of antibodies, the preponderance of these singly-bound biradicals disappears at the expense of doubly-bound biradicals, and the entire spectrum goes over into a (seemingly much weaker) strongly immobilized spectrum.

The above results demonstrate clearly the large effects that antibody-nitroxide spin label binding can have on paramagnetic resonance spectra; these effects involve a change of the hyperfine interaction, change of motion, and change of spin exchange interaction. It is likely that such effects will be useful for a variety of studies, particularly, of course, studies of the immune system itself.

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