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Magnetic Resonance of a Monoclonal Anti-Spin-Label Antibody†

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ABSTRACT: The nuclear magnetic resonance spectra of monoclonal Fab antibody fragments have been recorded in the absence and presence of a specific spin-label dinitrophenyl hapten. The difference spectra reveal the presence of 11–12 aromatic amino acids in the region of the combining site. By selective deuteration of this hybridoma antibody, these amino acids have been identified as three tryptophans, six or seven tyrosines, one phenylalanine, and one histidine. Difference spectra have also been recorded that depend on ring-current chemical shifts. The latter difference spectra show that among the 11–12 amino acids there are two tyrosines and one tryptophan in close proximity to the dinitrophenyl (DNP) ring. On the basis of ultraviolet absorption measurements, it is concluded that this tryptophan is stacked against the DNP ring. Selective deuteration of hybridoma antibodies directed against a paramagnetic hapten provides a powerful new approach for the study of the structural basis of antibody diversity and specificity.

Antibody molecules play a crucial role in defense against infection. When confronted by almost any foreign molecule, the immune system is able to produce antibody proteins of high affinity and exquisite specificity. The structural basis of antibody specificity has been one of the major concerns of immunochemistry for many years. Statistical analysis of antibody sequences shows that this specificity is determined by relatively short segments, the hypervariable regions (Wu & Kabat, 1970). Crystallographic studies reveal that the combining sites are formed by these hypervariable regions [for recent reviews, see Amzel & Poljak (1979) and Pecht (1982)]. Most of the amino acids in the nonhypervariable region form a rigid, relatively invariant framework, termed the immunoglobulin fold. This framework consists of two layers of antiparallel β-pleated sheets held together by a disulfide bond and enclosing a hydrophobic interior.

Complexes formed between haptens and specifically elicited anti-hapten antibodies have not yet been investigated by X-ray crystallography or nuclear magnetic resonance (NMR)[1]

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[1] Abbreviations: Fab, antigen binding fragment of antibody; SL, spin-label (see Materials and Methods for structure); DNP, dinitrophenyl; DNP-Gly, (2,4-dinitrophenyl)glycine; EDTA, ethylenediaminetetraacetic acid; NO, nitroxide; Fv, variable-region fragment of antibody; FabSL and FabDNP-Gly, complexes of antibody with indicated antigen; Tris, tris(hydroxymethyl)aminomethane. D(amino acid) means protein was grown with indicated amino acids deuterated; H(amino acid) means that the spectral features are mainly from the indicated amino acid.

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techniques (Pecht, 1982). However, by extensive screening, adventitious ligands have been found for many myeloma antibodies. Two complexes between myeloma proteins and such ligands have been studied by X-ray crystallography (Padlan et al., 1976a,b; Amzel et al., 1974). One of them was also studied by NMR (Goetze & Richards, 1978; Kooistra & Richards, 1978). In another study, Dwek and co-workers (Dwek et al., 1977; Dower & Dwek, 1979) studied extensively the MOPC 315–DNP complex by NMR. In early studies, these investigators attached a spin-label nitroxide group (SL) to DNP (Dwek et al., 1975). By taking the difference between the NMR spectra of the Fv fragment of the antibody and the FvDNP-SL complex, the resonances of protons within a radius of about 15 Å from the unpaired electron showed up in the difference spectrum. By taking the difference between the spectrum of the Fv–DNP complex and the spectrum of the Fv fragment, the only resonances that appear are those that undergo a chemical shift upon the binding of the hapten. These investigators found that this difference spectrum represents changes of the chemical shifts of about thirty protons. Dwek's studies rely heavily on a model for the binding site built by Padlan et al. (1976a,b), using extensive data obtained from kinetic mapping of binding (Pecht et al., 1972a,b) and results from affinity labeling (Givol et al., 1971; Goetzl & Metzger, 1970a,b). In an effort to improve assignments of proton resonance signals, Gettins & Dwek (1981) fed a mouse producing MOPC 315 a diet containing deuterated tryptophan.

The development of hybridoma technology makes it possible to obtain large amounts of chemically homogeneous antibody of known specificity (Kohler & Milstein, 1975). The eliciting antigen can be chosen to be a spectroscopically useful probe of its interactions with the antibody [for discussions of paramagnetic probes, see Likhtenstein (1974), Nezlin (1982), and Jardetzky (1981)]. Selective incorporation of deuterated amino acids is also possible, and this can be used to simplify the NMR spectra (Markley et al., 1968).

Our study shows that the broadening effects of the spin-label on the NMR spectrum of the protein can be combined with selective in vitro incorporation of deuterated amino acids to provide extensive information about the residues in and around the binding site. The ease of assignment of the numerous high-resolution resonances reported here suggests that further development may lead to structural information on combining sites of comparable quality to that obtainable from X-ray crystallography.

Materials and Methods

Deuterated Amino Acids. The following amino acids were obtained from MSD Isotopes: L-tryptophan-2,4,5,6,7-d_5 (96.92% D), L-β-(4-hydroxyphenyl-d_4)alanine-2,3,3-d_3 (tyrosine) (96.93% D), and L-phenyl-d_5-alanine-3,3-d_2 (98% D).

Cell Line. The AN02 cell line was derived in this laboratory and has been described previously (Balakrishnan et al., 1982). This cell line was adapted to growth in 1% serum and was recloned for high production. Typical yields are approximately 20 mg of antibody purified from 1 L of supernatant.

For growth on deuterated amino acids, 25 mL of medium [RPMI 1640, 1% fetal bovine serum, 1 mM glutamine, 1 mM pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin (Gibco)] with the appropriate deuterated amino acid (MSD Isotope), substituted for the protonated one, was added to a confluent monolayer of cells and incubated overnight. The medium was removed and discarded, and 75–100 mL of medium was then added to begin production. Supernatants were then removed every 3 days and replaced with fresh medium. Antibody yield is not noticeably affected by the presence of deuterated amino acids. For H(Tyr), a 3-fold excess of protonated tyrosine was used to prevent isotope incorporation from the deuterated phenylalanine.

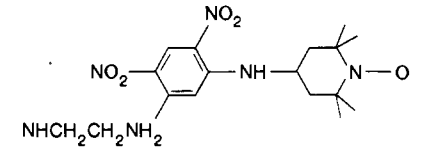
Sample Preparation. Cell supernatant was adjusted to pH 9 by the addition of Tris base and chromatographed on protein A–Sepharose (Pharmacia). The column was washed with pH 8.4 Tris- (50 mM) buffered saline and eluted with pH 4.7 citrate (0.1 M). The antibody was dialysis concentrated (collodion bag) against 0.1 M phosphate buffer, pH 7.2, containing 4 mM EDTA to a final volume of 5 mL. Papain (Sigma; 1% relative to antibody) was added; the mixture was made 0.01 M in cysteine and incubated for 4 h at 37 °C (Porter, 1959). The digestion was quenched by chromatography on Sephadex G-50 (80 × 2 cm) followed immediately by protein A–Sepharose. The Fab fragment was then concentrated to 10–15 mg/mL (assuming 1.4 absorbance units mg^{-1} mL^{-1}) by low-pressure dialysis (collodion bag). The concentrated sample was dialyzed against six changes of 50 mL of 0.01 M phosphate-buffered saline in doubly distilled deuterated water. Readings of pH are not corrected for isotope effects.

NMR Spectra. All spectra were taken on a modified Bruker 360-MHz spectrometer. Free induction decays were collected in 4000 data points after 60° pulses. Delays were 1 s with a sweep width of 3000 Hz. The number of scans taken per sample varied from 4000 to 8000 (1.5–3 h). The sample volume was 300 μL.

Spectra of the aromatic region were normalized relative to the maximum peak amplitude in the aliphatic region. Signal integrations for aromatic protons were made consistent between different spectra of a given type (Fab – FabSL, FabDNP-Gly – FabSL, etc.) by comparison with integrals of prominent signals in the aliphatic region for spectra of the same type.

ESR Spectra. These spectra were obtained on a Varian spectrometer (E102) with a 50-μL sample, under 15-mW power and 5-G modulation. The spectrum did not sharpen at a lower modulation amplitude.

Spin-Label Hapten. The following hapten was prepared as described previously (Balakrishnan et al., 1982):



Results

In the present work, we measured the binding of the spin-label hapten (DNP-SL) as well as DNP-Gly to AN02 antibody. The binding constant was determined from tryptophan fluorescence quenching. The results are displayed in Figure 1. The dissociation constants for DNP-SL and DNP-Gly are 2.5×10^{-7} and 5×10^{-7} M, respectively. These data are displayed here to show that there is no evidence for more than one binding constant. Low-affinity or heterogeneous binding can have significant implications for the interpretation of spin-label-broadened NMR spectra (Morisett et al., 1973).

The paramagnetic resonance spectrum of FabSL shown in Figure 2 confirms that the nitroxide group is in the combining site and has a rotational correlation time of 2×10^{-8} s (McCalley et al., 1972). That is, the motional freedom of the nitroxide group is no greater than that expected for the entire Fab molecule (Berliner, 1976).

The optical absorption spectrum of DNP-SL was measured in the presence and absence of AN02. The protein shifts the

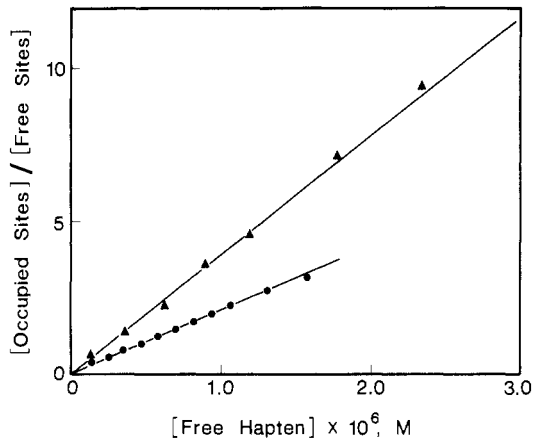


FIGURE 1: Binding of DNP-Gly (●) and SL (▲) to the antibody as a function of free hapten concentration. The ratio between the occupied sites and the free sites is obtained by fluorescence-quenching measurements (Eisen, 1964). Measurements were corrected for nonspecific quenching (not due to binding) and absorption by comparison with titrations of mouse IgG and of free tryptophan. Both corrections give the same results.

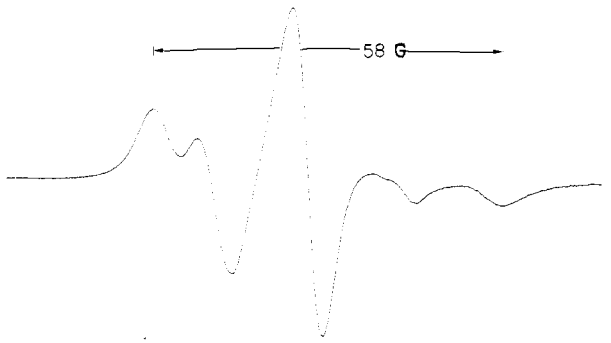


FIGURE 2: Paramagnetic resonance spectrum of FabSL. Fab concentration was 2×10^{-4} M; SL concentration was 1×10^{-4} M. This spectrum shows that the motional freedom of the nitroxide group of the spin-label hapten is no greater than that of the Fab molecule itself and that the rotational correlation time is of the order of 2×10^{-8} s.

near-UV peak of DNP-SL from 340 to 366 nm. This shift is attributed to charge-transfer interaction between DNP and tryptophan and is characteristic of most anti-DNP antibodies (Little & Eisen, 1967).

We found that the cell line AN02 is easily adapted to growth on deuterated amino acids and combinations of deuterated amino acids. In Figure 3, the symbols D(Trp), D(Trp,Phe), etc. refer to Fab antibody fragments derived from cell supernatants of AN02 grown on deuterated tryptophan, deuteriotryptophan and deuteriophenylalanine, etc. It can be seen from the spectra in Figure 3 that increasing deuteration leads to the expected decrease in the aromatic proton resonance intensity. The spectrum D(Tyr,Trp,Phe) shows essentially complete (>95%) elimination of the aromatic proton signals, except for His. (Evidence for highly efficient incorporation is also provided by comparison of single- and double-difference spectra discussed later.) We attribute this efficient incorporation of the deuterated amino acids to the fact that the AN02 cells were preadapted to growth on a low concentration (1%) of fetal bovine serum.

The signal to noise and reproducibility in the resonance spectra are sufficiently high to allow the calculation of informative difference and double-difference spectra. Figure 4 shows the difference spectra Fab – FabSL, i.e., the difference between the proton resonance spectrum of an Fab fragment and the spectrum of an Fab fragment having the spin label

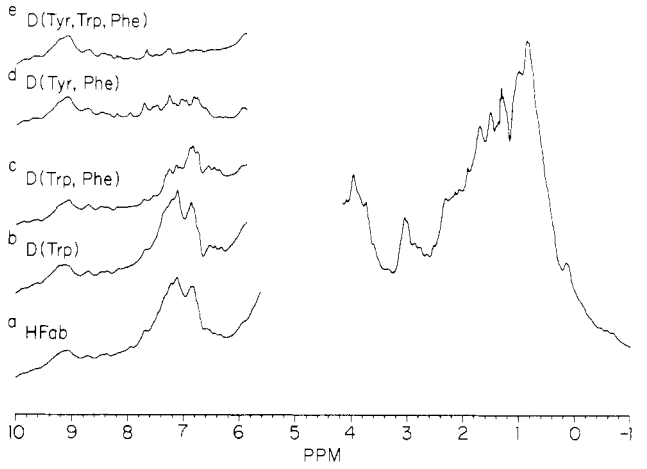


FIGURE 3: Spectra of Fab fragments containing indicated deuterated amino acids. Spectra are normalized with respect to the peak intensity in the aliphatic region at approximately 1 ppm (see Materials and Methods).

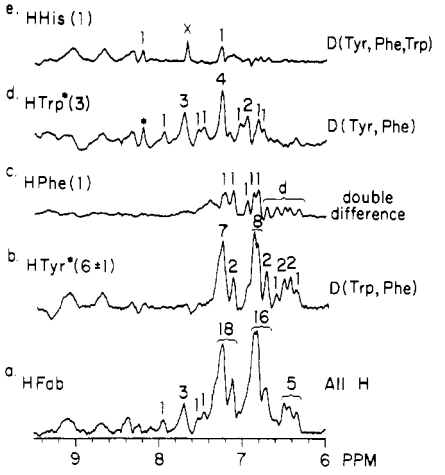


FIGURE 4: Spectrum of Fab minus spectrum of Fab with bound spin-label hapten bound (FabSL) for various deuterated proteins. Deuterated amino acids are indicated at the right; major contributions to proton resonances are at the left, followed by number of residues inferred. H(Trp)* and H(Tyr)* contain the two single resonances from histidine; H(Phe) does not, since it is a double-difference spectrum (see Results). Resonances labeled d are beyond the full broadening range of the spin-label and are called "distant".

hapten in the combining site. Such difference spectra lead to proton resonance signals from the region of the combining site due to nondeuterated amino acids. Figure 4 gives the isolated proton resonance signals for each aromatic amino acid in the combining site. Numbers above peaks indicate the number of protons. Two points are to be noted. The proton spectra of Trp and Tyr, denoted H(Trp)* and H(Tyr)* in the figure, contain the His resonances seen in the H(His) spectrum as well as amide proton resonance signals downfield from 8.35 ppm. In addition, the spectrum H(Phe) was obtained as a double difference, as discussed later. As expected, this spectrum does not contain the H(His) and amide protons. From integration of these spectra, we estimate that there are one His, three Trp, one Phe, and six or seven Tyr in the combining site. The numbers above the various signals in Figures 4 and 5 represent our best estimates for the number of protons responsible for each signal. The numbers for H-(Trp) were assigned on the assumption that the five relatively well-resolved low-field signals in Figure 5 arise from individual protons. Numbers above signals in other spectra are based on intensities relative to the tryptophan spectrum.

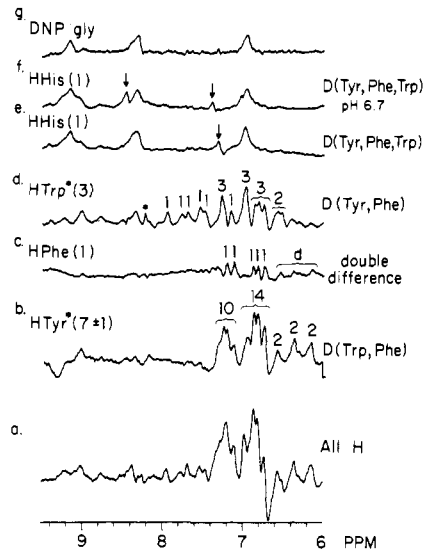


FIGURE 5: Spectrum of Fab minus spectrum of Fab with DNP-Gly bound (FabDNP-Gly) for various deuterated proteins. Labels are as in Figure 3. Top spectrum gives the three proton resonances of the DNP protons obtained by taking the difference between the spectrum of Fab in the presence of a 5-fold excess of DNP-Gly and the spectrum of Fab in the presence of a 2.5-fold excess of DNP-Gly.

The signal denoted X in the proton resonance of His in Figure 4 disappears after long dialysis against D_2O and probably arises from a difference in proton–deuteron exchange rate of His between FabSL and Fab. Conformation-dependent proton exchange rates are known for other proteins (Englander et al., 1972; Kim & Baldwin, 1982).

Detail. The H(His) difference spectrum in Figure 4 contains three signals from His protons, while the difference spectrum in Figure 5 contains only two His signals whose chemical shifts are very close to, if not identical with the chemical shifts of two of the three resonances in Figure 4. The two downfield His signals in Figure 4 are exchangeable protons (C2 H) as observed after the labeled Fab was in D_2O for 2 months. The upfield His signal is due to the nonexchangeable proton (His C4 H). Since there is only one C4 H His proton in the two difference spectra and one C2 H proton appears in both of them, we conclude that the X signal in Figure 2 does not arise from His proton (C2 H) in the vicinity of the unpaired electron but rather from an enhanced exchange rate in the FabSL and in FabDNP-Gly complexes.

Difference spectra of the type FabDNP-Gly – FabSL in Figure 5 represent aromatic proton signals from the combining site in the occupied conformation, plus the three aromatic proton resonances from DNP. There should then be no negative signals if the chemical shifts due to the two haptens were identical in the two spectra. The spectra indicate that this idealization is a reasonable but not perfect approximation.

The top spectrum in Figure 5 gives the difference in resonance spectra of Fab in the presence of a 5-fold excess of DNP-Gly and Fab in the presence of a 2.5-fold excess of DNP-Gly. The three proton signals from DNP are easily seen. These signals are of the order of 50 times broader than the signals of DNP-Gly in the absence of Fab. This enhanced and apparently homogeneous line width arises from rapid chemical exchange of DNP-Gly between the free and bound state. All of the other spectra in Figure 5 are from samples with only a small excess (≤20%) of DNP-Gly, except for the sample giving the H(His) spectra. In these two spectra, the arrows refer to His proton signals. The spectrum at pH 6.7 is given to display a His proton signal that is hidden by the DNP-Gly signal at pH 7.0.

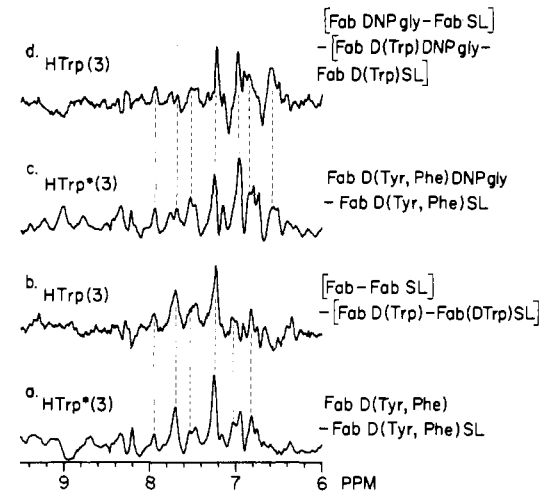


FIGURE 6: Single- vs. double-difference spectra: (a) single-difference spectrum Fab – FabSL when a selected amino acid is protonated; (b) double-difference spectra where Fab – FabSL for fully protonated and Fab – FabSL for Fab deuterated at a selected amino acid are subtracted; (c and d) FabDNP-Gly in place of Fab.

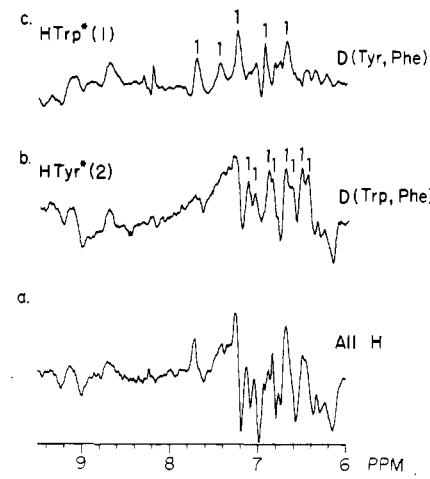


FIGURE 7: Fab – FabDNP-Gly for various deuterated proteins. Signals in spectra b and c arise from protons in tyrosine and tryptophan that are in close contact with the DNP ring.

The overall concordance of the spectra in Figures 4 and 5 is very good in terms of the number of protons assigned to each aromatic amino acid in the combining-site region. In a number of cases, individual proton signals have the same chemical shift in both types of spectra. Various details of these spectra will be discussed later.

Figure 6 gives a comparison between single difference spectra such as Fab[D(Tyr,Phe)] – Fab[D(Tyr,Phe)]SL and double-difference spectra such as (Fab – FabSL) – (Fab[D-(Trp)] – Fab[D(Trp)]SL). Note that both difference spectra should give the H(Trp) proton signal in the binding site region, except for the small H(His) signal and amide protons in the single-difference spectrum. The excellent concordance of these spectra is significant since the two types of difference spectra are based on independent experimental measurements. The observed larger noise in the double-difference spectrum is expected for statistical reasons. Good agreement is also to be seen in the comparable single- and double-difference spectra in Figure 6 when the spectra of FabDNP-Gly and FabSL are employed. For these reasons we are confident of the double-difference spectra of H(Phe) given in Figures 4 and 5.

The difference spectra shown in Figure 7 can also be used to provide useful information on the amino acid composition of the binding site, especially for amino acids whose resonances

are shifted by close proximity to the bound DNP ring system. Correlation of signals in these spectra with the appropriate spectra in Figures 4 and 5 allows an estimate of the number of protons responsible for their appearance. For example, in the D(Tyr,Phe) difference spectrum in Figure 7, the feature at 7.3 ppm must arise from one proton since the corresponding features in Figures 4 and 5 have integrals of four and three protons, respectively. In the D(Trp,Phe) difference spectra in Figure 7, eight individual proton signals from tyrosine have been assigned as indicated.

Discussion

The present paper describes an approach to the study of the structure of antibody combining sites. Our work represents a significant improvement over previous applications of nuclear resonance and spin-labels to this problem in that (i) the monoclonal antibody studied is specific for the paramagnetic hapten, (ii) the in vitro hybridoma culture is found to allow selective, highly efficient incorporation of deuterated amino acids, and (iii) the appropriate difference spectra provide direct assignments of resonance signals arising from amino acids in the combining-site region.

In this study, all protons within a distance of the order of 15 Å from the paramagnetic NO group are believed to undergo resonance line broadening to the point that their signals appear at full strength in the difference spectra. (The signals indicated by the symbol d in Figures 4 and 5 are thought to be at distances somewhat greater than 15 Å from the NO group.) For typical packing densities of amino acids in globular proteins, one can estimate that there may be as many as 40 amino acids in a sphere having a radius of 15 Å. Clearly, the 11 aromatic amino acids identified in the present study constitute only a fraction of all the amino acids whose spectra are affected by the unpaired electron. Studies in progress in this laboratory demonstrate the presence of aliphatic amino acids in this same region. An important problem is the identification of the amino acids that are in direct contact with the hapten. In the present work, a *single* Trp residue and two Tyr residues are strongly implicated as being in contact with the DNP portion of the hapten. This Trp residue is responsible for the charge-transfer interaction that affects the UV spectrum of DNP.

Relative distances of various amino acids from the paramagnetic NO group are currently being determined by employing difference spectra for solutions containing various lower concentrations of the hapten. The hapten chemical exchange rate is rapid so that one can vary the distances over which the combining-site proton resonance signals are broadened. The present work opens a clear path for a study of the structural diversity of monoclonal antibodies against spin-label haptens.

Acknowledgments

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Registry No. L-Tryptophan, 73-22-3; L-tyrosine, 60-18-4; L-phenylalanine, 63-91-2; L-histidine, 71-00-1.

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