greater in older age groups and is increasingly so in the most recent year period population.

Not only is the mean age at diagnosis increasing, but the rate of *incidence* is rising much more rapidly in the older age groups as time goes on.

In cancer of the skin where increased stimuli from external environmental agents of theoretically possible carcinogenic potentialities are encountered throughout life, the trend is toward an increase in earlier ages.

In cancer of the digestive organs and peritoneum, where internal biochemical changes are considered as being of potential carcinogenic significance, this is not the case. Whatever slight trend there may be is in the direction of increased relative incidence in older age groups.

In cancer of the lung and bronchus, the trend and the increasing rate of incidence are strikingly toward the older age groups, or in directly the opposite direction from that of skin cancer.

In conclusion, it should be emphasized that existing data on the increase or decrease of age-corrected incidence of cancer of different sites are of a secondary order of scientific exactness. They do, however, indicate interesting and specific trends which need further study and analysis.

The author wishes to acknowledge the great assistance of Mrs. Rita Simpson in tabulating and charting the data.

[1] *Cancer in Connecticut 1935–51*, Conn. State Dept. of Health (1955).

[2] *Cancer in New York State 1941–60*, Bureau of Cancer Control, N.Y. Dept. of Health (1962).

[3] *United States Census of Population 1940*, U. S. Govt. Printing Office (1942).

[4] Both the trends of change in total number of cases and of "age-corrected" death rates per 100,000 will be discussed. More detailed presentation of data on "age-corrected" death rates will be attempted because these data discount any actual numerical shifts in the age-distribution of the various populations.

[5] The data on cancer of the buccal cavity and pharynx were not based on large numbers, since the disease is not common. They showed no very marked trends. Cancer of the prostate showed slight trend toward older age groups.

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*A SPIN-LABELED HAPTEN**

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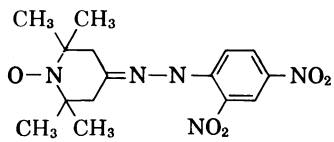
The use of stable, organic, free radical labels in studies of biological macromolecules has recently been introduced by Ohnishi and McConnell.[1] Subsequently, Stone, Buckman, Nordio, and McConnell[2] carried out an electron spin resonance (ESR) investigation of a nitroxide radical covalently linked to serum albumin and to poly-L-lysine, and obtained a measure of the rotational mobility of the region of the macromolecule to which the spin labels were bonded. In the present study, we use a free radical hapten to probe the antibody combining site. The hapten (I)

consists of a 2,4-dinitrophenyl moiety against which the antibody specificity is directed and a nitroxide radical group which provides the unpaired electron spin:



I

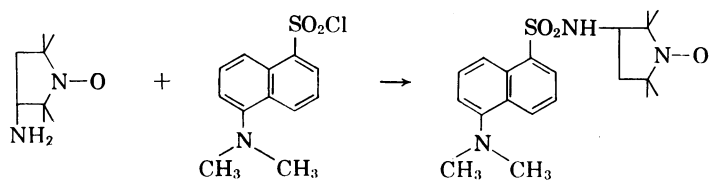
This compound was first synthesized by Rozantzev and Neiman[3] as a derivative of a ketone nitroxide. We find that this molecule binds with high affinity to antibody directed against the dinitrophenyl group (antidinitrophenyl antibody). We report here fluorescence and ESR studies of the hapten-antibody interaction. These studies have provided a measure of the rigidity of the antibody combining site.

Experimental.—Dinitrophenyl nitroxide (I): The 2,4-dinitrophenyl hydrazone of 2,2,6,6-tetramethyl-4-piperidone nitrogen oxide was prepared by the method of Rozantzev and Neiman.[3] One gm of the ketone (20% excess) was dissolved in water and added with stirring to one liter of 20% phosphoric acid containing 1.0 gm of 2,4-dinitrophenylhydrazine. The orange precipitate was washed, dried, and recrystallized twice from an ethanol-ethyl acetate solution.

Anal: Calc. for $C_{15}H_{20}H_5O_2$: C, 51.4; H, 5.8; N, 20.0%. Found: C, 51.4; H, 6.0; N, 20.1%.

The absorption maxima of I in 10% ethanol–90% H_2O occur at 230 and 368 mμ, where the ϵ are 17,000 and 22,600 cm^2/mmole, respectively.

Dansyl nitroxide (IV): 2,2,5,5-Tetramethyl-3-amino-pyrrolidine-1-oxyl (II) was prepared by the procedure of Rozantzev and Krinitzkaya.[4] In 1 ml reagent grade acetone 0.20 gm II (20% excess) was added to a solution of 0.28 gm 1-dimethylaminonaphthalene-5-sulfonyl chloride (III, obtained from Aldrich Chemical Co.) in 5 ml acetone. The solution was stored for 2 hr at 30°C and the acetone was removed *in vacuo*. The yellow solid was dissolved in ether and extracted



II III IV

repeatedly with a pH 6.8 aqueous buffer to remove unreacted amine (II). The ether extract was chromatographed on a silica column and was eluted with acetone. The resulting dansyl nitroxide (IV), 1-dimethylamino-naphthalene-5-(N-1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-sulfonamide, contains 6.0 ($\pm$0.2) $\times$ 10^{23} spins/mole. The absorption spectrum of IV in ethanol exhibits maxima at 251 and 337 mμ, with extinction coefficients of 15,000 and 4,200 cm^2/mmole, respectively. An ϵ of 4,300 cm^2/mmole at 337 mμ is typical of dansyl sulfonamides.[5] A broad emission peak with a maximum at 530 mμ was observed for the dansyl nitroxide in 90% glycerol–5% H_2O–5% ethanol at 25°C.

Anti-2,4-dinitrophenyl antibody was kindly provided by Mrs. Diane Griffin of Stanford Medical School. The antibody was obtained from rabbits immunized against 2,4-dinitrophenylated hemocyanin. Dinitrophenylated bovine γ-globulin was used to precipitate the antibody. Antibody concentrations were based on a specific absorption coefficient of 1.46 cm^2/mg and a molecular weight of 160,000.[6]

Fluorescence measurements: Fluorescence titrations and fluorescence polarization measurements were carried out as previously described.[7] The excited state lifetime of dansyl nitroxide was determined on a nanosecond flash apparatus constructed by Mr. Lee Hundley of the Medical School Instrumentation Research Laboratory, supported in part by NASA grant NsG-81-60. The nanosecond fluorimeter and data processing system will be described in detail elsewhere.[8]

ESR spectra were recorded on a Varian 9.5 Gc/sec spectrometer. All spectra were recorded as first derivatives of the ESR absorption.

Results.—Fluorimetric determination of the binding of dinitrophenyl nitroxide to antidinitrophenyl antibody: Dinitrophenyl nitroxide binds strongly to antidinitrophenyl antibody, as shown by a fluorescence titration (Fig. 1). The fluorescence quenching method of Velick, Parker, and Eisen[6] was used to determine the stoichiometry of binding and obtain an estimate of the equilibrium constant. The interaction of the dinitrophenyl nitroxide with the antibody was compared with that of a standard hapten, ϵ-N-dinitrophenyl-L-lysine.[6] The titration curves for these two haptens (Fig. 1) are very similar. Both haptens bind with an equilibrium constant less than 10^{-8} M. At equivalence, nearly two hapten molecules are bound per antibody (molecular weight 160,000). It therefore appears that the nitroxide ring does not interfere with the specific, high-affinity interaction of the dinitrophenyl group with the antibody. Fluorescence measurements show that nonspecific γ-globulin does not bind dinitrophenyl nitroxide.

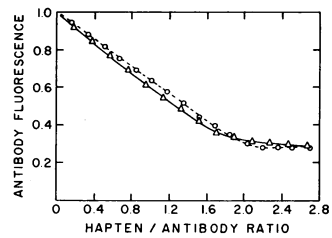
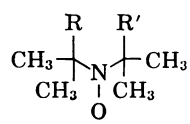


FIG. 1.—Fluorescence titration of the binding of hapten to antidinitrophenyl antibody. Aliquots of dinitrophenyl nitroxide (Δ–Δ–Δ) or of dinitrophenyl-L-lysine (O–O–O) were added to 1.6×10^{-7} M antibody in 0.1 M phosphate buffer, pH 6.8. The antibody fluorescence was excited at 280 mμ and observed at 350 mμ. The equivalence point of the titration corresponds to nearly 2 haptens per antibody of molecular weight 160,000.

ESR spectra of dinitrophenyl nitroxide: The ESR spectra of dinitrophenyl nitroxide in the presence and absence of antidinitrophenyl antibody are shown in Figure 2 (*A* and *B*, respectively). *The striking change in the ESR spectrum upon addition of the antibody clearly indicates that the tumbling motion of the nitroxide free radical is markedly reduced upon formation of the antibody-hapten complex.*

The dinitrophenyl nitroxide radical is a member of the important class of nitroxide radicals having the general structure



Several authors have observed the ESR spectra of this type of radical.[3, 4, 9] The approximate spin Hamiltonian, $\mathcal{H}$, is given by

$$\mathcal{H} = \mathcal{H}_z + hAS_zI_z + hBS_xI_x + hCS_yI_y,$$

where $\mathcal{H}_z$ is the electron Zeeman Hamiltonian, and A, B, and C represent the sum of the Fermi contact term and the electron-nuclear dipolar term along the three principal axes of the nitroxide radical.[10] Griffith, Cornell, and McConnell[11] have studied nitroxide radicals oriented in a diamagnetic crystalline host and find that the largest splitting ($A \sim 87$ Mc/sec) occurs with the magnetic field along the π-orbital of the nitrogen atom, while the splitting is much smaller and nearly isotropic ($B \simeq C \simeq 14$ Mc/sec) in the xy plane of the radical. In the rapidly tumbling case this anisotropy is, of course, averaged to zero and the splitting is given by the Fermi contact component $|a| = {}^1/_3 |A + B + C|$. In a rigid glass spectrum the two outermost lines are separated by the maximum possible splitting, $2A$. A

rigid EPA glass spectrum and a typical solution spectrum of dinitrophenyl nitroxide are given in Figure 2*C* and *D*, respectively. It is apparent from the spectrum in Figure 2*A* that the rotational motion of the bound dinitrophenyl nitroxide is highly restricted but not as immobile as nitroxide in a rigid glass.

Stoichiometry: Further information may be obtained from the ESR spectra by varying the hapten/antibody ratio. The spectra for 1.30 haptens/antibody (Fig. 2*A*) and 1.80 haptens/antibody (Fig. 3*A*) are similar, except for two very weak signals indicated by the arrows, which arise from a trace of unbound hapten in the latter solution. The spectrum of 2.28 haptens/antibody (Figs. 3*B* and *C*) is markedly different due to the predominance of the ESR signal from unbound hapten. The sharpness and high peak intensity of the three lines seen in the spectrum

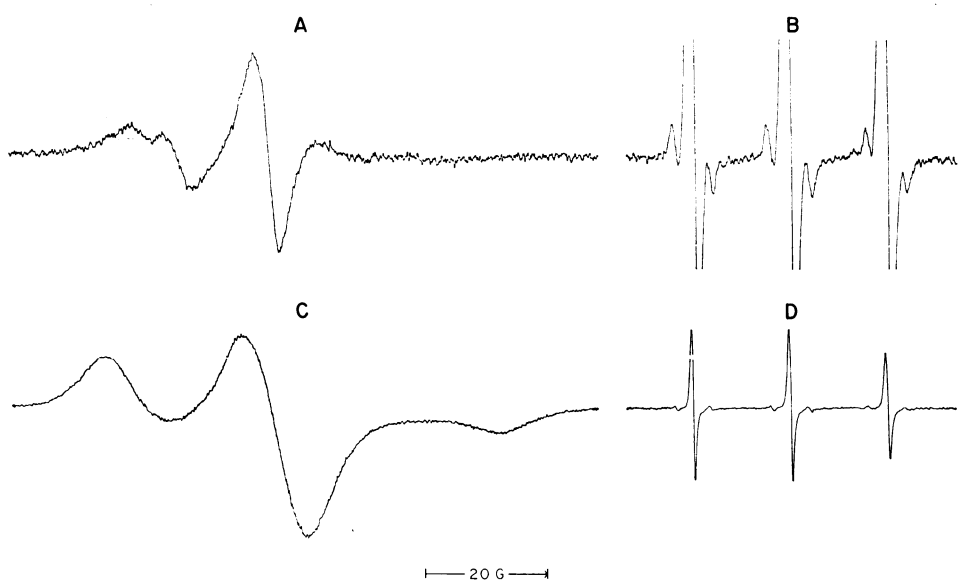


FIG. 2.—ESR spectra of dinitrophenyl nitroxide: (*A*) in aqueous solution containing antidinitrophenyl antibody; (*B*) and (*D*), in aqueous solution without antibody; (*C*) in EPA glass at −196°C. The spectrometer sensitivity is the same for (*A*) and (*B*), and tenfold lower for (*D*). The sensitivity of (*C*) is not quantitatively related to the others. The free radical concentrations of (*A*), (*B*), and (*D*) are 10^{-4} *M*, while that of (*C*) is 10^{-3} *M*. The antibody concentration in (*A*) is 0.77×10^{-4} *M*. Aqueous solutions were in 0.1 *M* phosphate buffer, pH 6.8, at 23°C.

of rapidly tumbling dinitrophenyl nitroxide contrast with the broad spectrum observed for the slowly rotating free radical, thus making it feasible to detect small amounts of unbound hapten. The height of the central peak of the ESR spectrum of unbound hapten is about an order of magnitude greater than that of bound hapten. This large change in spectrum clearly identifies the stoichiometry of interaction between the hapten and antibody, in good agreement with the value of about 2.0 haptens/antibody obtained from the fluorescence titration (Fig. 1).

Estimate of the rotational relaxation time of the bound hapten: The most direct way of determining ρ, the rotational relaxation time, is to match the experimentally observed ESR spectrum of the bound hapten (Fig. 2*A*) with theoretical spectra computed for various values of ρ. However, the present density matrix treatments are not readily applicable to this range of tumbling frequencies.[12, 13] It is therefore

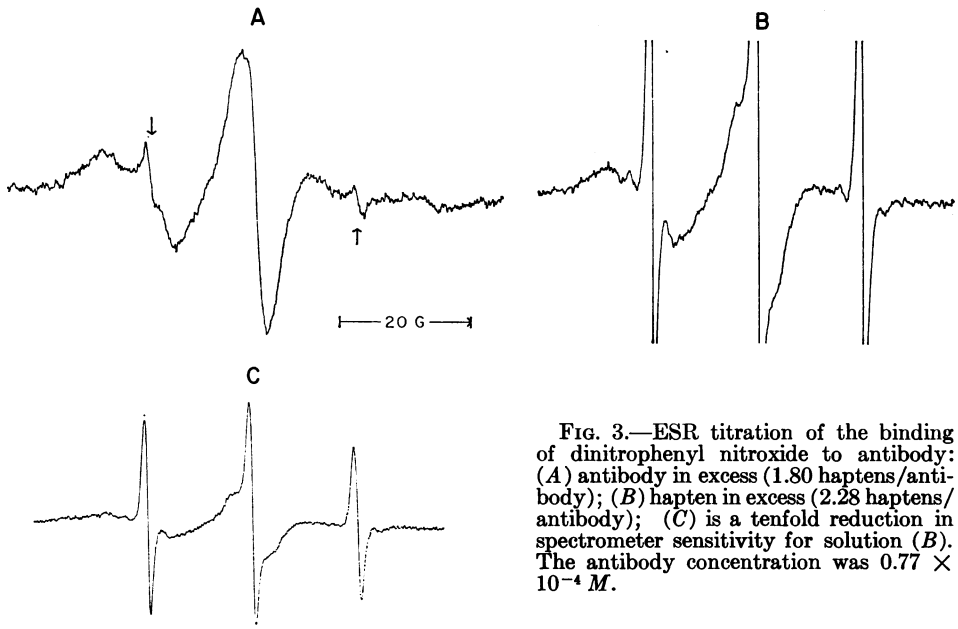


FIG. 3.—ESR titration of the binding of dinitrophenyl nitroxide to antibody: (*A*) antibody in excess (1.80 haptens/antibody); (*B*) hapten in excess (2.28 haptens/antibody); (*C*) is a tenfold reduction in spectrometer sensitivity for solution (*B*). The antibody concentration was 0.77×10^{-4} *M*.

of some interest to explore indirect methods of obtaining estimates of the rotational relaxation time. One such method involves a combination of ESR and fluorescence measurements of a molecule containing both a fluorescence-label and a spin-label. Dansyl nitroxide (IV) was used for this purpose. First, ESR spectra of dansyl nitroxide in aqueous glycerol solution with viscosities ranging from 0.01 to more than 2 poise (Fig. 4) were compared with the spectrum of dinitrophenyl nitroxide bound to antibody (Fig. 2*A*). The best correspondence was obtained with dansyl nitroxide in 90 per cent glycerol–5 per cent H_2O–5 per cent ethanol at 35°C (Fig. 4*C*). Then, the fluorescence polarization of this matching solution was measured in order to obtain an estimate of the rotational relaxation time.

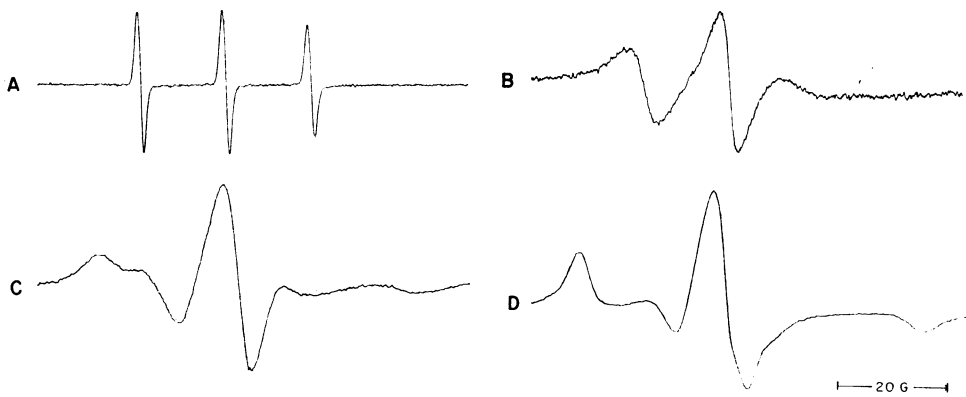


FIG. 4.—ESR spectra of dansyl nitroxide in solutions of increasing viscosity: (*A*) 95% H_2O–5% ethanol at 23°C; (*B*) 76% glycerol–19% H_2O–5% ethanol at 23°C; (*C*) 90% glycerol–5% H_2O–5% ethanol at 35°C; (*D*) 90% glycerol–5% H_2O–5% ethanol at −15°C. The ESR spectrum of solution (*C*) corresponds most closely to the spectrum obtained for dinitrophenyl nitroxide bound to antibody (Fig. 2*A*). Ethanol (5%) was used to increase the solubility of dansyl nitroxide in aqueous glycerol.

This approach involves a number of assumptions, in addition to the usual one of spherical averaging. First, it assumes that the rotational relaxation time of the nitroxide portion of the dansyl nitroxide is identical to that of its fluorescent dansyl group. Molecular models indicate that the rotation about the sulfonamide bond is definitely hindered, making this approximation a reasonably valid one. Second, it assumes that the principal elements of the electron-nuclear hyperfine matrix and of the g-tensor are nearly the same for dansyl nitroxide and dinitrophenyl nitroxide. Anisotropic g-tensor data are not yet available for these two radicals. However, the measured isotropic g-values for nitroxide radicals of this type are nearly identical.[11] In addition, the splittings observed for solutions of these two radicals are approximately the same, indicating that their Fermi contact terms are nearly equal. It is particularly significant that the rigid EPA glass spectra of these two free radicals are virtually superposable. This finding emphasizes that the largest hyperfine splitting terms, as well as the principal g-values, are nearly identical for the two radicals. The second assumption, therefore, is also likely to be in large measure valid.

The rotational relaxation time, ρ, is related to the fluorescence polarization by the expression $\rho = 3\tau A/A_0 - A$, where τ is the excited state lifetime and A_0 is the emission anisotropy in the absence of molecular rotation.[5] The emission anisotropy[14] is defined as $A = I_x - I_y/I_x + 2I_y$, where I_x and I_y are the intensities of x- and y-polarized fluorescence obtained with x-polarized excitation. The observed emission anisotropy of a 10^{-4} M solution of dansyl nitroxide in 90 per cent glycerol-5 per cent H_2O-5 per cent ethanol at 35°C, excited at 380 mμ, was 0.162. A value of 0.320 for A_0 was determined by measuring the emission anisotropy of dansyl nitroxide in a low-temperature glycerol glass. An identical value of A_0 was obtained by an extrapolation to $T/\eta = 0$ in a linear plot of $1/A$ versus T/η, for η from 0.1 to 5 poise. The fluorescence decay kinetics following nanosecond pulse excitation corresponded to an excited state lifetime of 11.7 nsec. Using $A = 0.162$, $A_0 = 0.320$, and $\tau = 11.7$ nsec, a rotation relaxation time of 36 nsec was obtained for the dansyl nitroxide in 90 per cent glycerol-5 per cent H_2O-5 per cent ethanol at 35°C.

The accuracy of this estimated rotational relaxation time is not yet known, since it was obtained by an indirect method.[15] It is of interest that the estimated ρ of 36 nsec is significantly shorter than the value of 200 nsec obtained by Krause and O'Konski[16] from electric birefringence studies of γ-globulin. The difference might reflect the uncertainty of our estimate or, more likely, it may indicate that there is a small degree of rotational flexibility of the nitroxide radical relative to the whole antibody. Molecular models of the hapten do not exclude the possibility that there may be some free rotation of the nitroxide ring relative to the dinitrophenyl group. The relaxation time estimated here sets a lower limit on the size of the rotationally rigid unit with which the hapten interacts. It is therefore evident from the present study that the bound hapten rotates in common with a high molecular weight segment of the antibody and that the antibody combining site has little flexibility when the hapten is bound.

Summary.—Dinitrophenyl nitroxide, a spin-labeled hapten, binds with high affinity to antidinitrophenyl antibody. An electron spin resonance study of their interaction has provided a measure of the rotational mobility of the hapten when bound to antibody. We find that this antibody combining site is characterized by a

high degree of rigidity. The stoichiometry of interaction between hapten and antibody was determined by an electron spin resonance titration, in agreement with the results obtained from a fluorescence titration. It appears likely that the spin-labeling method will be useful in studies of the interaction of haptens, coenzymes, inhibitors, and substrates with proteins and other macromolecules.

We are indebted to Professor Harden M. McConnell for numerous stimulating discussions and encouragement in this work. We wish to thank Mrs. Diane Griffin for her gift of antibody. It is a pleasure to acknowledge the capable technical assistance of Miss Verena Kurer.

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*IN VITRO STUDIES ON THE MECHANISM OF SUPPRESSION OF A NONSENSE MUTATION**

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THE ROCKEFELLER UNIVERSITY AND YALE UNIVERSITY

Communicated by E. L. Tatum, November 5, 1965

On the basis of genetic and physiological analyses, Benzer and Champe[1] and Garen and Siddiqi[2] concluded that suppression of nonsense mutations occurred at the level of translation of messenger RNA into protein. It remained to be shown which, if any, of the known components of protein synthesis were responsible for suppression. The present report describes the use of an *in vitro* assay for suppression to identify a suppressor component. The development of this assay depended on the following observations. (1) Unsuppressed nonsense mutations cause the premature termina-