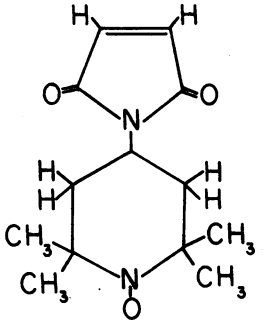
*SPIN-LABELED NUCLEIC ACIDS**

By Ian C. P. Smith† and Tetsuo Yamane

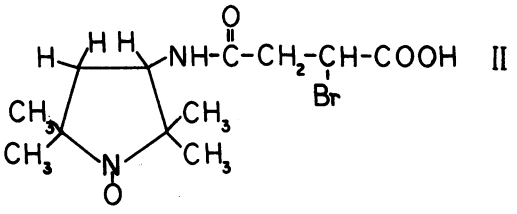
BELL TELEPHONE LABORATORIES, INCORPORATED, MURRAY HILL, NEW JERSEY

Communicated by Harden M. McConnell, July 20, 1967

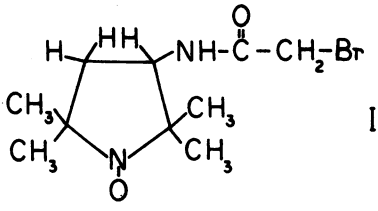
Recently the technique of spin labeling has been applied to study the conformational properties of biological macromolecules.[1-7] Up to now, however, the application has been limited primarily to polypeptides. We wish to report the use of the three compounds below to spin-label polynucleotides.



I



II



III

Compound I was synthesized by Dr. J. C. A. Boeyens[4] according to the method of Hamilton.[5] It has the advantage over the five-membered nitroxide-maleimide spin label[2] in that it is not subject to rapid hydrolysis. Compound II was prepared during a study on the conformational properties of the active side of RNase A.[7] Compound III was synthesized according to the procedure described by Ogawa and McConnell.[6] The EPR spectra of the buffered aqueous samples were taken at room temperature on a Varian V-4500 X-band spectrometer.

All three labels reacted with ribonucleic acid at pH 5.5 in 0.1 *M* acetate buffer at 25°C. Excess spin label was removed by exhaustive dialysis. The product of reaction between I and RNA (Calbiochem, highly polymerized, from yeast) gave an EPR spectrum corresponding to a mobile spin label attached to a large molecule (similar to Fig. 1 but without the broad components between the principal lines). Addition of pancreatic RNase A resulted immediately in a narrowing of the three EPR lines (similar to Fig. 2) and a shift in their relative peak-to-peak intensities towards the 1:1:1 expected for a freely rotating nitroxide molecule. Compound II reacted with RNA to yield a similar EPR spectrum which was sensitive to RNase A. In both cases the estimated per cent labeling was less than 1 per cent.

Compound III reacted most strongly with RNA (approximately 5% labeling),

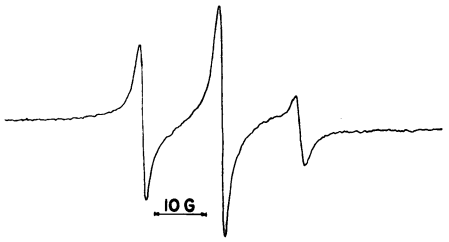


FIG. 1.—EPR spectrum of highly polymerized RNA spin-labeled with the bromoamide label III at pH 5.5. The separation between the principal hyperfine lines is 15.9 gauss.

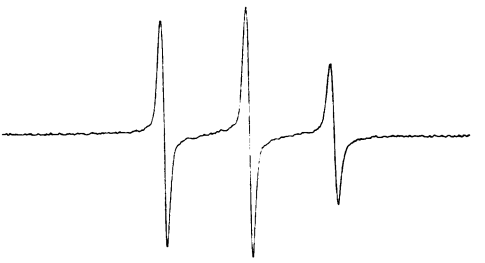


FIG. 2.—EPR spectrum of sample responsible for Fig. 1, after treatment with pancreatic ribonuclease. Scale same as Fig. 1.

and the resulting EPR spectrum is presented in Figure 1. Of particular interest in this spectrum are the broad components between the principal lines. This type of spectrum indicates attachment to a region of the RNA molecule with sufficient secondary structure to impede rotational averaging of the spin-label hyperfine and g tensor anisotropy. Addition of RNase A resulted in disappearance of the broad spectral components and a narrowing of the three principal lines (Fig. 2).

Reaction of the three labels with unfractionated sRNA (yeast, General Biochemicals) gave results identical to those just described for the highly polymerized yeast RNA.

All three spin labels reacted weakly with deoxyribonucleic acid (Calbiochem and Worthington, calf thymus) at pH 5.5 to yield spectra similar to that in Figure 3*a*, i.e., with two components, one corresponding to an immobilized spin label, and another corresponding to a spin label with partly restricted rotation. The relative areas of the two spectral components indicate that the reaction resulting in a highly restricted environment for the spin label occurs to the greater extent (approximately five times greater).

Because compound III reacted with RNA and DNA more strongly than I or II, its behavior with respect to the nucleotide bases and synthetic polynucleotides was studied in detail. Reaction of III with polyguanylic acid (Miles Laboratories) at pH 5.5 in 0.1 M acetate buffer resulted in an EPR spectrum corresponding to an immobilized spin label. This is indicative of the high degree of secondary structure thought to exist in poly G at low pH.[9] At higher pH an EPR spectral component corresponding to a more mobile spin label appeared (Fig. 3*a*). Further increase in pH resulted in gradual conversion of the broad EPR lines to their nar-

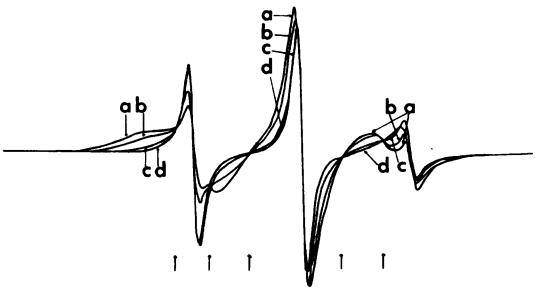


FIG. 3.—Superposition of traces of EPR spectra due to poly G, labeled with III, at (*a*) pH 10.45, (*b*) pH 10.95, (*c*) pH 11.60, (*d*) pH 11.80. The solid arrows at the bottom of the figure indicate isobestic points. Scale same as Fig. 1.

rower counterparts (Fig. 3*b–d*). By following the relative amounts of the two types of spectrum it is possible to measure pK for the transition of poly G from a highly ordered to a disordered conformation. The EPR data yields pK = 11.5 ± 0.1, which is in agreement with the value of 11.43 reported by Pochon and Michelson[9] for poly G in 0.15 *M* NaCl. It can be seen from Figure 3 that several isobestic points are present in the superposition of EPR spectra taken at different pH values; they are indicated by the vertical arrows at the bottom of the figure. The presence of isobestic points demonstrates that the spin label in labeled poly G is present in only two environments, and implies that there are only two distinct conformations of poly G in solution (on the time scale of the EPR method). It should be pointed out that since the poly G is only weakly spin-labeled (in order to avoid extrinsic effects due to labeling), the EPR method is monitoring only a small portion of the total molecule. The correspondence of the pK values obtained by EPR and optical methods lends support to the conclusion that poly G exists in only two forms under the conditions of these experiments.

Polyadenylic acid (Miles Laboratories) reacted with III to yield EPR spectra similar to those obtained with poly G. At pH 5.5 the spin label was completely immobilized, and the transition from the ordered to disordered state occurring at higher pH values could be followed by the relative amounts of broad and narrow EPR spectral components. The pK observed for this transition is 5.9 ± 0.1, in agreement with the value of 6.0 obtained by optical methods.[8, 12] Superposition of the EPR spectra at various pH values over the range 5.5 to 7.5 yielded three isobestic points, indicating that poly A exists in only two conformations under these conditions. Labeling poly A at pH 7.5 yielded a more intense EPR spectrum, but the pH dependence was identical to that of poly A labeled at pH 5.5.

Polyuridylic acid (Miles Laboratories) spin-labeled with III at pH 5.5 gave an EPR spectrum similar to that of Figure 2, indicating that the polynucleotide exists primarily in a flexible random coil conformation. However, reaction of III with polycytidylic acid (Miles Laboratories) at pH 5.5 resulted in an EPR spectrum similar to that in Figure 3*a*. This implies that at pH 5.5 in 0.1 *M* acetate buffer poly C retains a highly ordered structure, which is consistent with other experimental data.[10, 11]

In order to establish that the bases in RNA and DNA were labeled by III, adenine, guanine, cytosine, uracil, and thymine were reacted with III in acetate buffer pH 5.5. The individual mixtures were then chromatographed on a column of Dowex 1-X2 using a gradient of ammonium formate (0.03 *M*, pH 10, to 0.36 *M*, pH 5.0); the column effluent was monitored by optical absorption at 260 mμ and by electron spin resonance absorption. In all cases evidence for a spin-labeled product was obtained, and with thymine two spin-labeled compounds of considerably different column mobilities resulted. The order of appearance from the column was: compound III, C, T-III, C-III, U-III, T, A-III, U, T-III′, A, G, G-III. The extent of reaction was: G, 20%; A, 3%; C, U, and T, less than 1%.

The foregoing data indicate that the spin-labeling technique may be gainfully applied to biological problems involving nucleic acids. Of particular interest is the preferential reaction of III with adenine and guanine—presumably because the alkylating agent has a much higher reactivity towards nitrogen-7 of the purines

relative to the available sites of reaction in the pyrimidine bases—and the observation that both poly A and poly G exist in only two forms in the pH region of the transition from a highly ordered to a disordered conformation. The immobilization of label III by polynucleotides with considerable secondary structure (poly A, poly G, poly C, sRNA, DNA) provides a sensitive probe of such structure.

It is a pleasure to acknowledge that this problem was suggested by Professor Harden M. McConnell.

* The first portion of this work was carried out in the Stauffer Laboratory of Physical Chemistry at Stanford University, and was sponsored in part by the Office of Naval Research under contract 255(88), 1965–1966.

† NATO postdoctoral fellow, 1965–1966, Stanford University. Permanent address: Division of Pure Chemistry, National Research Council, Ottawa, Canada.

[1] Stone, T. J., T. Buckman, P. L. Nordio, and H. M. McConnell, these PROCEEDINGS, **54**, 1010 (1965).

[2] Griffith, O. H., and H. M. McConnell, these PROCEEDINGS, **55**, 8 (1965).

[3] Berliner, L. J., and H. M. McConnell, these PROCEEDINGS, **55**, 708 (1966).

[4] Ohnishi, S., J. C. A. Boeyens, and H. M. McConnell, these PROCEEDINGS, **56**, 809 (1966).

[5] Hamilton, C. L., unpublished results.

[6] Ogawa, S., and H. M. McConnell, these PROCEEDINGS, **58**, 19 (1967).

[7] Smith, I. C. P., and H. M. McConnell, in preparation.

[8] Beers, R., and R. Steiner, *Nature*, **179**, 1076 (1957).

[9] Pochon, F., and A. M. Michelson, these PROCEEDINGS, **53**, 1425 (1965).

[10] Lipsett, M. N., *J. Biol. Chem.*, **239**, 1256 (1964).

[11] Fasman, G. D., C. Lindblow, and L. Grossman, *Biochemistry*, **3**, 1015 (1964).

[12] Rahn, R. O., T. Yamane, T. Eisinger, T. W. Longworth, and R. G. Shulman, *J. Chem. Phys.*, **45**, 2947 (1966).