# NMR Analysis of DNA Junctions: Imino Proton NMR Studies of Individual Arms and Intact Junction†

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ABSTRACT: The NMR resonances from the hydrogen-bonded guanine and thymine imino protons of base pairs in the four separate complexes forming the arms of a stable DNA four-arm junction have been assigned by using sequential nuclear Overhauser effects connecting protons in adjacent pairs. Comparison of the spectra of these individual duplex arms with that of the intact four-stranded junction suggests that base pairing occurs at the site of branching. The presence of new resonances in the spectrum of the junction can be inferred from comparison of the junction spectrum with the simulated spectra of the four individual arms. In addition, upfield shifts of the ring protons in the base pairs at the penultimate positions in the complex are observed, consistent with a change in the structure at the site of branching. These studies represent the first stage of a detailed analysis of the structure and dynamics of a DNA junction.

The application of high-resolution $^1$H NMR spectroscopy to DNA fragments and tRNA molecules has been dramatically enhanced by the use of nuclear Overhauser effects (NOEs) connecting one base pair in a duplex to its next neighbors (Roy & Redfield, 1981). The low-field spectral region, below about 10 ppm from sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS), includes resonances from the exchangeable ring N–H's of T and G in duplexes with hydrogen-bonded A-T (U) and G-C base pairs (Shimmel & Redfield, 1980). These protons provide useful probes of the structure and dynamics of nucleic acid double helices in solution (Reid, 1981; Johnston & Redfield, 1981; Patel et al., 1982b). We have recently reported on the design, synthesis, and experimental characterization of a unique class of nucleic acid structures, DNA junctions, in which three or more strands interact via complementary base pairing to form stable branched complexes (Seeman, 1982; Seeman & Kallenbach, 1983; Kallenbach et al., 1983a). Such junction structures occur naturally during recombination of two homologous DNA sequences and in cruciforms generated in supercoiled DNA plasmids. Although the primary structure of such junctions, i.e., the regions of base pairing, is understood, there is almost no information about their structure on a microscopic level. A number of models for a four-strand junction have been proposed (Sigal & Alberts, 1972; Sobell, 1972; Wilson, 1979; Seeman et al., 1979); however, there is no direct support for any of them. There is also considerable interest in the dynamics of migration of these junctions along symmetric or near-symmetric DNA sequences. From the dynamic models (Seeman & Robinson, 1981), it is clear that a great deal more information about the structure is required before the dynamic questions can be settled in detail. The preparation of immobile junctions now makes it possible to examine the conformations, energetics, and dynamics in a system where the signal from the site of branching is a detectable component in that of the complex as a whole (Kallenbach et al., 1983b).

We have previously reported that the four strands shown in Figure 1 form a stoichiometric complex in solution, with distinctive electrophoretic mobility in polyacrylamide gels (Kallenbach et al., 1983a). We present here a $^1$H NMR study of the hydrogen-bonded ring imino proton resonances of T and G in the individually paired arms forming this complex and show by comparison with the spectrum of the four-strand structure that base pairing at the site of branching can be detected. This comparison also indicates that resonances from imino protons of base pairs next to those at the junction center shift upfield in the complex, consistent with pairing at the site of branching.

## Materials and Methods

The four hexadecanucleotide strands with sequences illustrated in Figure 1 were synthesized by P-L Laboratories, Milwaukee, WI, using phosphotriester techniques. The sequences were selected by an algorithm designed to favor formation of a tetrameric complex by minimizing competing Watson–Crick pairing alternatives among the four strands (Seeman & Kallenbach, 1983). According to the rules applied, branch migration of the central branch point has been eliminated by forbidding sequence symmetry around the junction; thus, the tetramer will not resolve itself into linear duplexes by this isomerization reaction (Seeman, 1982).

NMR spectra were run on samples containing 2 mM DNA in solutions containing 100% $D_2O$ for locking, 10 mM phosphate buffer, pH 7, and 5 mM $MgCl_2$. The spectrometer used was a Bruker WM 500, operating at 500 MHz for protons. The Redfield 214 pulse sequences was applied to suppress the solvent signal (Redfield, 1978). NOE difference spectra were obtained by interleaving free induction decays (FIDs) accumulated with the decoupler off-resonance with FIDs recorded with the decoupler on a line of interest. Imino resonances were irradiated for 0.4–0.8 s for NOE buildup. All spectra were resolution enhanced by Gaussian multiplication.

## Results

The imino proton region of the $^1$H NMR spectrum of the tetrameric complex with equimolar concentration of the strands labeled 1–4 in Figure 1 is shown in Figure 2 at a series of

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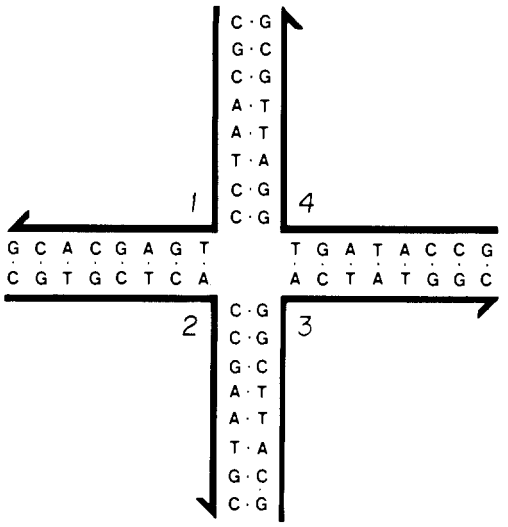


FIGURE 1: Immobile nucleic acid junction composed of four hexadecanucleotides. This sequence has been designed by using sequence symmetry minimization, supplemented by equilibrium distribution calculations (Seeman, 1982; Seeman & Kallenbach, 1983). Note the lack of 2-fold symmetry around the center, so that branch point migration is not possible. The strand numbering is used throughout the text. This sequence also contains no repeating GpG sequence longer than two, in order to minimize potential non-Watson–Crick pairing.

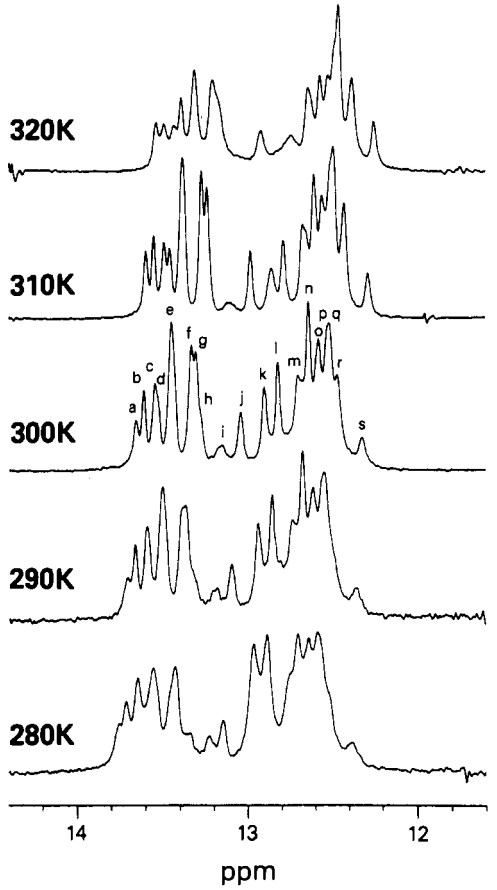


FIGURE 2: $^1$H NMR spectra of the junction complex at several temperatures. Loss of resolution due to increased line widths at low temperature can be seen. Shifts in frequency of the low-field set of resonances relative to the cluster at higher field are observed.

temperatures below the denaturation temperature of the complex (observed optically to be at ca. 65 °C). Resonances from imino protons in oligonucleotide duplexes are observed to broaden with increasing temperature, due to an increase in the exchange rate with solvent protons (Hilbers, 1979).

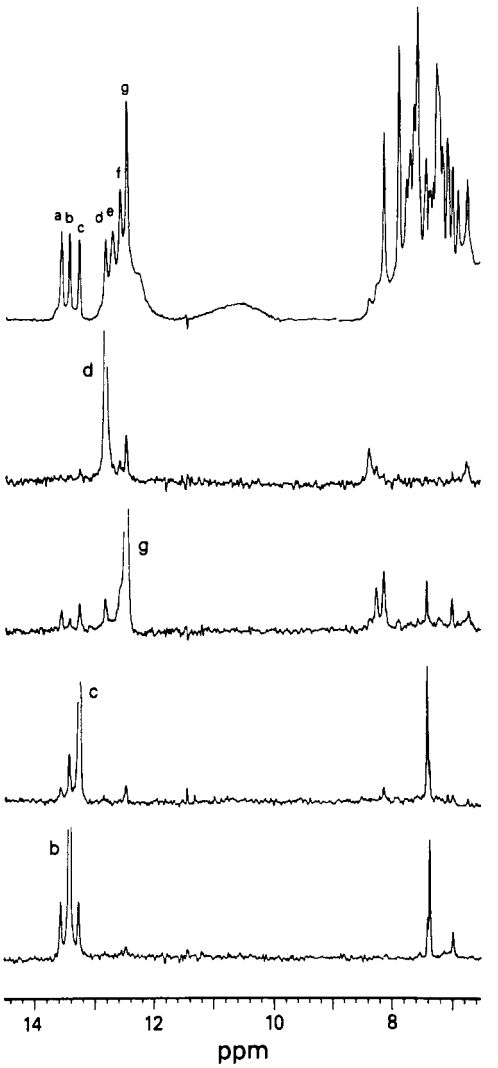


FIGURE 3: Assignment of imino resonances in the complex of strands 2 + 3. The top spectrum shows the low-field $^1$H spectrum of an equimolar mixture of strands of 2 and 3 in Figure 1 at 290 K. The four spectra below present some NOE difference spectra corresponding to irradiation of peaks d, g, c, and b of the reference spectrum.

It has been shown from gel electrophoresis that the complementary pairs of strands in Figure 1 form duplexes on mixing (Kallenbach et al., 1983a). The imino resonances of the four individual arms provide a means of interpreting the complex spectrum of the junction itself. By use of selective NOE experiments, the imino resonances in the individual paired arms of the tetramer can be assigned quite straightforwardly, since the spectra of these are well resolved at 500 MHz. Our approach to assigning resonances in the spectra of the tetramer is then to assume conservation in chemical shifts between internal base pairs of the individual arms in their free state and as they exist, in the junction complex (Figure 1). These assignments can then be verified or negated by comparison with NOEs determined on the intact junction.

Resonance assignments in the individual arms can be made from the NOEs from the imino protons of one base pair to proton on adjacent pairs in a double helix (Roy & Redfield, 1981; Chou et al., 1983). Figure 3 illustrates the appplication of this procedure to the assignment of the imino protons of the complex involving strands 2 and 3 (arm 2 of the junction). We saturated in turn each imino resonance of the reference spectrum shown in Figure 3 in order to establish the identity of the base pairs giving rise to each peak. Only A-T imino protons have an adjacent C(2)–H to cross-saturate; this gives

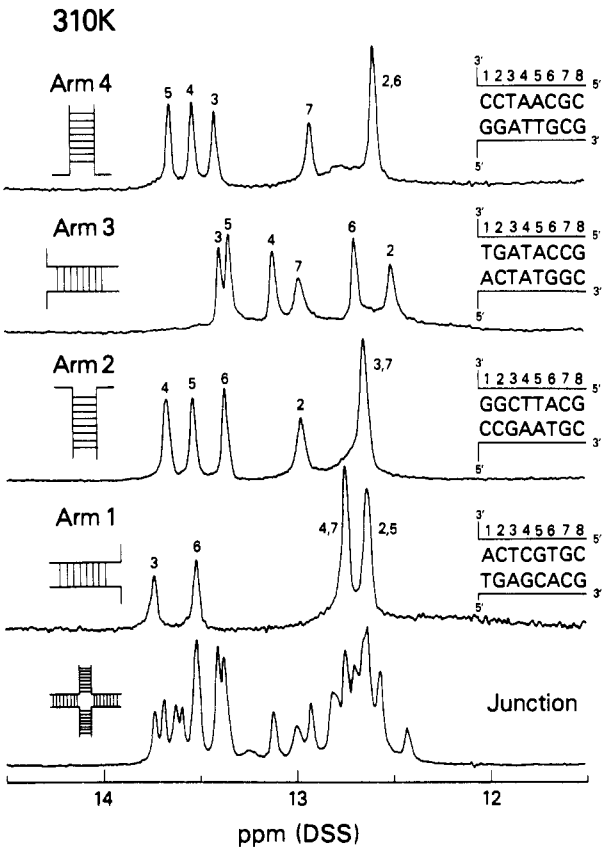


FIGURE 4: Summary of assignments of pairwise complexes of the strands. At 310 K, resonances corresponding to six internal imino protons in each arm are detected. These are numbered as shown in the inserts at the right, with 1 representing the position closest to the junction. The inserts on the left of each spectrum indicate the position of each arm within the junction, in accord with the orientation shown in Figure 1.

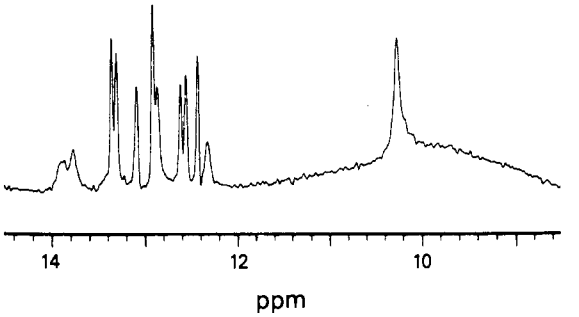


FIGURE 5: Spectrum of the complex of an equimolar mixture of strands 3 and 4 (arm 3) at 290 K. This spectrum is included to illustrate nonstandard base pair formation at low temperatures, which occurs in at least two of the arms. The nature of the additional peaks near 10 and 14 ppm has not yet been established. The former region is characteristic of looped-out thymine imino protons (Haasnoot et al., 1983).

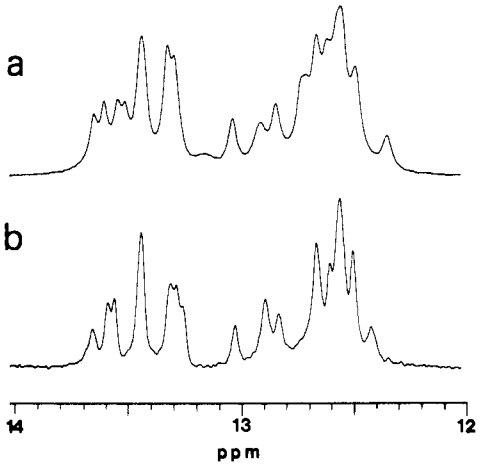


FIGURE 6: Comparison of the spectrum of the intact junction with the summed spectra of the pair complexes of the four arms. (b) Algebraic sum of the four spectra in Figure 4, with appropriate resolution enhancement to more closely match the line width of the junction. (a) Spectrum of the intact junction at 310 K.

rise to a characteristic strong NOE to a narrow line in the aromatic region. Peaks a, b, and c show this behavior, while peaks d, e, f, and g do not. Hence, we assign a, b, and c as peaks arising from A-T base pairs and d, e, and g as peaks from G-C base pairs.

The NOE from peak b to both peak a and peak c (bottom spectrum, Figure 3) indicates that peak b corresponds to base pair 5, since this is the only A-T base pair in the sequence with two flanking A-T neighbors. Similarly, peak d shows an NOE only to G-C resonances, peak g, and not to A-T pairs. Hence, we assign peak d to the imino proton of G-C resonance 2. Peak g, on the other hand, shows connectivity to both peak a and peak c. This situation is consistent with assignment of resonances a and c as A-T base pairs 4 and 6, G-C resonances 3 and 7 both residing in peak g. Identification of peak a with A-T base pair 4, rather than base pair 6, is based on chemical shift, since its neighbors are pyrimidines rather than purines. The latter give rise to stronger upfield shifts as nearest neighbors (Arter & Schmidt, 1976). Similar chemical shift arguments and analysis of imino to C(2)–H NOEs (Chou et al., 1983) were also required, in addition to the directly observed NOEs, for assignment of arms 1 and 4.

Figure 4 summarizes the results of applying this procedure to each of the four arms of the junction. As can be seen from the figure, the resonances from the base pairs at either end of the double-helical region are not seen at 310 K. Data were collected at this temperature in order to avoid pairing from the parts of the 16-mers which are not Watson–Crick complementary. An example of a low-temperature spectrum showing extra resonances from the noncomplementary sequence of the strand is shown in Figure 5. At least 13 resonances can be seen, corresponding to the 16-mer which has an 8 base pair section of complementary, with those outside the complementary region showing greater thermolability than those within. Similar extra resonances were also seen in other arms. Octamers corresponding to just the complementary regions are currently being synthesized; these will facilitate assignments of the additional base pair resonances at lower temperatures and the terminal base pairs of the complementary section.

The synthetic spectrum of the junction, generated by summing the spectra of all the arms, is contrasted with the spectrum of the intact junction in Figure 6. The algebraic sum of the four spectra corresponding to the arms, with less resolution enhancement than used for the full junction spectrum to better match line widths, is shown at the bottom. The spectrum of the intact junction, also at 310 K, is shown above the summed spectrum. There are several points worth noting in this comparison. Many of the peaks have not shifted signifiantly in formation of the junction from the separate arms, suggesting that the resonances which do shift are those near the juction site itself. Several peaks which correspond to the base pairs penultimate to the branch point shift upfield in the complex, consistent with a change in stacking with the end base pairs. For example, the furthest upfield peak, at about 12.4 ppm, in the junction spectrum can be identified as the penultimate G-C resonance in arm 3, shifted upfield by about

0.1 ppm relative to arm 3 alone. Similarly, the peak at about 13.0 ppm in arm 2, corresponding to its penultimate base pair, appears to shift upfield by at least 0.2 ppm.

In addition, several new resonances are observed which do not correspond with any observed in the arms at the same temperature. Three clear peaks near 13.6, 13.25, and 12.8 ppm arise from new (stabilized relative to the arms alone) base pairs at the branch point of the junction. These can be identified by comparison of the number of lines in the individual arm spectra (Figure 4) with the number near the same chemical shift in the intact junction. In the 300 K spectrum of Figure 2, these new lines correspond to peaks c or d, i, and one of the peaks in m. By comparison with Figure 4, we conclude that the other peak of c or d arises from base pair 5 of arm 4. With the possibility of small shifts, we cannot unambiguously say which is which. The second peak in m is likely due to base pair 2 of arm 2, shifted upfield by improved stacking and stabilization in the intact junction. Careful simulations of the spectral region from 13.1 to 13.8 ppm show that 13 resonances occur in this range, while only 11 are accounted for by comparison with the arm spectra. The spectral region from 12.4 to 13.1 ppm was also simulated; however, the simulated spectrum was of lower intensity than the observed spectrum over the range 12.6–13.0 ppm. It is likely that this difference stems from the four terminal (position 8 in Figure 1) base pair resonances, which are strongly broadened by exchange with solvent at this temperature (compare also Figure 2, 280 K spectrum). To match the sharp features of the spectrum in this region, 15 resonances must be included, 13 of which are predicted from the arm spectra. This suggests that in this region also, there must be two new resonances in the junction spectrum arising from base pairs in arms 2 and 4 at the branch point. One of these, as noted above, is very probably in peak m; however, due to problems of intensity from the broad component, and the possibilities of shifts in other resonances, the other new peak cannot be clearly identified. The temperature dependence of the imino resonances in the junction is shown in Figure 2. The resonance near 13.2 ppm is fairly broad, even at 300 K, increasing further in width at higher temperature. The rate of increase in width with temperature, however, is less than that normally seen for central base pairs, suggesting a weak base pairing at the junction. An example of a "normal" temperature dependence is given by the resonance near 13.0 ppm, corresponding to base pair 7 of arm 3.

It is possible to establish the identity of some resonances in the junction directly using NOEs, examples of which are shown in Figure 7. For example, we confirm that the most upfield of the A-T base pairs in the junction corresponds to base pair 4 of arm 3, at the same chemical shift as observed in the arm alone. Unfortunately, due to the rather severe overlap of peaks for much of the imino region, it has not been possible to unambiguously assign all of the imino resonances. It is even more difficult for broadened peaks, such as the new resonance near 13.2 ppm, since the rapid relaxation makes the peak very difficult to saturate. Thus, it is difficult even to be certain if this resonance corresponds to a G-C or A-T base pair, though the latter seems much more likely in light of the discussion above.

## Discussion

Analysis of the imino resonances of the individual arms of the four-arm junction complex (Figure 1) provides evidence for formation at 310 K of several base pairs which abut the junction. Differences between the spectrum of the junction and the summed spectra of the isolated arms as pairwise

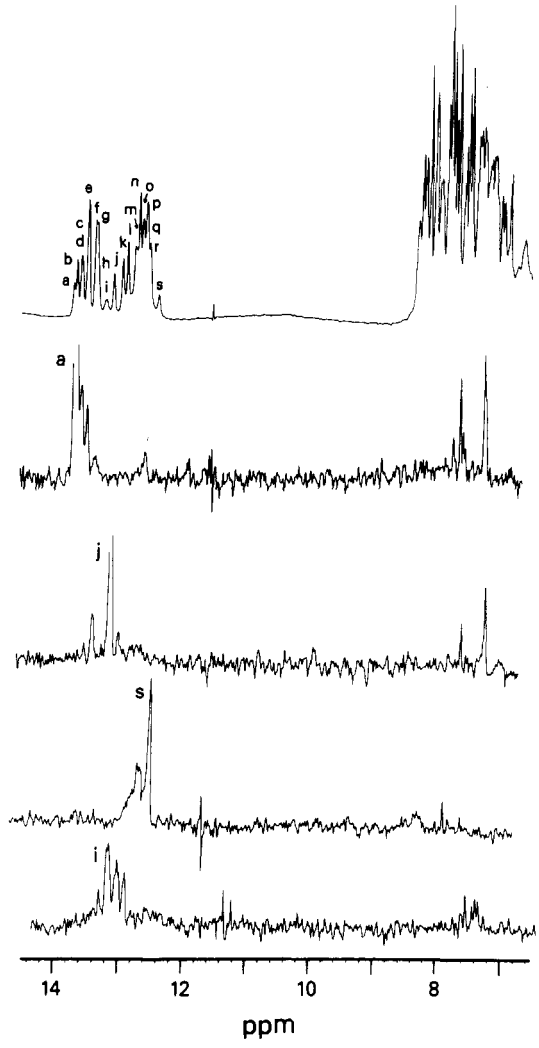


FIGURE 7: Sample NOE difference spectra of the junction complex at 300 K. The top spectrum shows the reference spectrum of the junction. The spectra below contain the NOE difference spectra obtained by irradiation of the indicated resonances in this spectrum.

complexes also occur in several regions of the spectrum, consistent with the presence of both A-T and G-C pairs, although we have no direct assignments of these. The upfield shifts observed for each of the resonances corresponding to imino protons of base pairs penultimate to the junction suggest that all the base pairs indicated in Figure 1 do form around the junction. This effect would arise from ring current shielding by the terminal bases, and possibly those across the branch point. The resonances which have been ascribed to the junction base pairs seem to vary significantly in chemical shift and line width (reflecting stability). Most of them are also shifted upfield from the chemical shift expected if they had neighboring base pairs only on one side, as terminal base pairs do. These facts suggest that there is a well-defined structure at the junction, although with present data it is not possible to give conformational details in this region. In other junction sequences, it appears there may be more than one stable conformation at the branch point (Kallenbach et al., 1983b). Further measurement of imino proton exchange rates for base pairs at or near the junction in mobile or semimoble junction sequences should provide dynamic information about the junction migration process. However, before these can be analyzed, assignments and more information about the structure near the branch point are required. The work described here begins such an analysis. In addition, we have shown that unusual base pairing in noncomplementary se-

quences can occur in sequences such as those in the arms of the junction.

It is important to point out that the NMR analysis of this junction is consistent with previous physical studies on the complex. Circular dichroism studies have indicated that the presence of the junction does not perturb the structure of the DNA distal to it (Seeman et al., 1984); the CD spectrum is characteristic of B DNA. The close correspondence seen between the spectra observed for the pairwise arm duplexes and the spectra of the complete intact junction is consistent with this observation. Thus, only the local neighborhood of the junction is likely to display unusual structural features.

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# Spectra, Membrane Binding, and Potentiometric Reponses of New Charge Shift Probes†

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ABSTRACT: The properties of a series of new potentiometric membrane probes have been explored. The probes all contain an (aminostyryl)pyridinium chromophore or a more highly conjugated analogue. The spectral properties of the dyes are discussed in terms of the excitation-induced charge shift from the pyridine to the aniline; this charge shift also provides the basis for the voltage dependence of the spectra according to an electrochromic mechanism. The spectral responses to a membrane potential on a hemispherical bilayer have been obtained and, grossly, are quite similar for all probes tested. The more subtle variations from dye to dye can be partially rationalized by consideration of binding parameters, the depth within the membrane, and structural factors. The most potential sensitive dye in this collection has been designated di-4-ANEPPS and has a 6-amino-2-naphthyl group in place of the *p*-anilino on the parent chromophore. Both the relative fluorescence emission and excitation responses have maxima of 8% per 100 mV, and these two spectra display a striking symmetry.

Optical potentiometric probes have become important tools in electrophysiology. These organic molecules display spectroscopic responses to membrane potential and have been used for the study and characterization of model membranes, nerve and muscle tissues, organelles, microorganisms, and red blood cells (Waggoner, 1979; Cohen & Salzberg, 1978; Freedman & Laris, 1981). They can often be used in place of conventional microelectrodes and lend themselves to many systems not accessible to microelectrodes. Our laboratory has concentrated on the development of probes that respond via an electrochromic mechanism (Loew et al., 1978; Loew, 1982).

In an electrochromic mechanism, molecules undergo an electronic redistribution upon excitation, and if the direction of the charge shift is parallel to an external field (membrane

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