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Publication Date

1972-10-01

Submitted to the Journal of
American Chemical Society

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October 1972

AEC Contract No. W-7405-eng-48

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PHOTOCHEMICALLY INDUCED DYNAMIC NUCLEAR POLARIZATION IN HYDROSOL SUSPENSIONS
OF CONGO RED*

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Abstract

Visible irradiation of dilute acidic hydrosol suspensions of Congo-red were found to sensitize chemically induced dynamic nuclear polarization of the solvent NMR. Neutral solutions of the dye did not produce the effect. Evidence is presented that the polarization is produced in the hydrosol particles and transferred by chemical exchange to the solvent. A mechanism for the photooxidation of Congo-red is postulated.

Introduction

Freshly formed products of free radical pairs show emission and enhanced absorption lines in their NMR spectra, a phenomenon which has been named "Chemical Induced Dynamic Nuclear Polarization (CIDNP).¹

A necessary condition for producing polarized NMR spectra of this sort is a competition between spin-dependent annihilation of the radical pair to form a singlet and spin-independent scattering to form the two separate radicals.² In trying to apply this technique to biological systems we are faced with the problem of predicting the behavior, when the participants of the radical pair will be high molecular weight macromolecules.

If the polarized protons are held rigidly to the macromolecule, their linewidths, $\Delta\nu$, will be the same as those on unpolarized molecules: a reasonable rule-of-thumb is that $\Delta\nu \sim MW \times 10^{-3}$ Hz. Thus macromolecules in the range of 50,000 - 100,000 should yield detectable spectra. If, by contrast, the polarized protons undergo rapid exchange with the solvent, the polarization could be monitored by observation of the relatively narrow solvent NMR lines.³

The current concepts of CIDNP have evolved from consideration of simple, homogenous solutions of small molecules.² The complexities which may be anticipated for application of CIDNP to biological materials are manifold, but at least two are readily apparent.

The first is that of a homogenous solution of macromolecules and the second is the inherent heterogeneity of any but the simplest biochemical systems.

Since the process is bimolecular, the rate constants are diffusion controlled as the relative diffusion coefficients of the constituents determine the lifetimes of the radical pairs. In simple solutions of small molecules this lifetime is shorter than the electron-electron exchange time, i.e., $10^{-9} - 10^{-10}$ sec vs. 10^{-8} sec.

For a simple solution of macromolecules where one or both of the fragments are small, a similar situation might be anticipated. If either fragment is large, however, then diffusion might be restricted, thus reversing the above situation.

Heterogenous systems contain all of the complexities noted, as well as others, known or unknown. This latter situation is the novel concern of this paper, in which we report that a polarization formed in macroscopic particles is transferred by chemical exchange to the solvent, where it was observed by high resolution NMR.

Experimental

All chemicals were commercially available and were used without further purifications. The pH was adjusted using NaOD or DCl. Absorption spectra were taken using a Cary 14 spectrophotometer. The NMR spectra were obtained at 60 MHz using a Varian A-60 spectrometer equipped with a light guide, as described earlier.⁴ The EPR spectra were obtained using a Varian E-3 spectrometer. EPR spectra in the liquid phase were taken using a flow system driven by a peristaltic pump. For both the NMR and EPR experiments, the light source was a Hanovia 1000 W high pressure mercury-xenon lamp in a Shoeffel housing. The light intensity dependence was measured with a series of calibrated mesh filters.

Results

Absorption Spectra. The absorption spectra of the acidic and neutral forms of dilute solutions of Congo-red (see formula in Scheme I) in H_2O are shown in Figure 1. The neutral form has its absorption maximum at 495 nm, while in the acidic form this maximum shifts to 565 nm. The color of the dye in these two forms changes from red to blue. The pK of the transition is around 4. Another marked difference between the acidic and the neutral form of Congo-red is that in the neutral form it forms a simple red solution, while in the acidic form it coagulates to form a hydrosol suspension. The light scattering which appears in the spectrum of the acidic form, shown in Figure 1, results from this coagulation.

NMR. The NMR spectrum of a 10^{-4} M acidic suspension of Congo-red in D_2O is shown in Figure 2B. Upon irradiation, the proton line of the 0.4% HOD of the solvent is completely inverted and enhanced (Figure 2A). D_2O solutions of the same concentration at neutral pD showed no polarization, nor did solutions in H_2O . The pH dependence is discussed in subsequent sections.

Among the many possible reasons for the absence of an observable polarization in the fully protonated solvent, we mention the most important. The steady state polarization is proportional to the proton T_1 ,^{2g} which is inversely proportional to the proton concentration in H_2O - D_2O mixtures.⁶

We calculated the polarization in the usual manner.

Figure 3 shows how the polarization varies with the light intensity. From the linear dependence we see that the rate of formation of the radical pair is proportional to the light intensity. Consequently

the radical pair must be formed from a single precursor. This is equivalent to stating that the polarization must originate in the initial photochemical act. Due to the low signal-to-noise, it was impossible to take proper action spectra for producing the polarization. Using Corning glass filters we found that about 50% of the polarization arises from actinic light with wavelength >400 nm. From the absorption spectrum appearing in Figure 1, the chromophoric group which is responsible for the 565 nm absorption peak must be directly involved in the polarization process.

EPR. The EPR of the system was examined to gain some insight into the nature of the radicals. For direct comparison with the NMR data, the first experiments were performed in the liquid phase. No radicals were detected in either the neutral or acidic forms, even after introducing various scavengers such as nitromethane, various aliphatic alcohols and acetone, which are known to react with small, short-lived radicals, to produce relatively long-lived radicals. Next, samples of both acid and neutral solutions of Congo-red were frozen, irradiated, and observed at 77°K.

Figure 4 gives the EPR spectrum of a frozen acidic solution. The neutral solution gave no observable free radicals. This finding confirmed qualitatively the NMR result, that a free radical photochemical reaction occurs in the acidic form, while none is observable in the neutral form. Any attempt to analyze the spectrum in Figure 4, using saturation study, partial thermal annihilation, and replacement of H_2O with D_2O as a solvent, failed. Thus, detailed chemical information about the nature of the radicals involved is not yet available.

Functional Group Analysis. An alternative way to get information about the nature of the intermediates involved was to test different compounds with the same functional groups. The compounds checked were: aniline, 3,5-diaminobenzoic acid, *p*-aminoacetophenone, 3-amino-2,7-naphthalenedisulfonic acid, benzene-azo- α -naphthylamine and methyl-red. These compounds cover the possible functional groups in Congo-red (see Scheme I for formulas), and all were checked over a large pH range. The last two in the list are also dyes which can serve as indicators, with a similar pH range. We observed no NMR polarization in any of these compounds. The only obvious difference between these compounds and Congo-red was that in the range of optical absorption involved (O.D. $\approx$ 2) all these compounds gave a true solution over the pH range studied, while Congo-red at acidic pH was in a coagulated form. The last observation gave us good reason to suspect that the NMR polarization and the photochemistry might be a solid state phenomena.

State of Aggregation. To check the last possibility, an acidic suspension was centrifugated at 20,000 rpm. The supernatant appeared clear and the resuspended pellet retained all the polarization. An alternative way to explain the last result is that upon irradiation the Congo-red gives some soluble photochemical product which absorbs the light and gives the polarization. We will return to this point later when dealing with the time evolution of the polarization. To check this possibility, the sample was irradiated outside the spectrometer and aliquots taken at various times. The supernatant and the washed pellet were centrifugated and checked. In all cases the supernatant gave no polarization, while the resuspended washed pellet

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retained its full polarization upon reillumination. To check further, the difference spectrum between identical fresh and 1 hour irradiated samples was measured. The spectrum is shown in Figure 1C. Less than 10% of the Congo-red disappeared during this time, while traces of some new compound with maximum absorption at 400 nm appeared. (The light intensity during the irradiations outside the NMR probe is much higher than that during the polarization experiments.) The observed polarization cannot be accounted for on the basis of the low optical density of the new compound. Besides, as mentioned before, about 50% of the polarization appeared with actinic light of $\lambda > 400$ nm. As a final proof for demonstrating that the polarization is indeed a solid state phenomena, the red neutral solution of Congo-red, which gave no polarization, was salted out with 5% NaCl and heated to boiling for 1 hour. (The same result could be achieved without heating, but would require a waiting period of a few days.) The supernatant from this solution was again clear. The precipitate was resuspended and the observed polarization was of the same order of magnitude as the original acidic solution.

If this polarization is a solid state phenomena, can it occur on a solid support? To analyze for this possibility, we attached the Congo-red to 0.1% of bovine serum albumin which was lyophilized from D₂O. The NMR spectra in the pH range of 3-7 showed no detectable polarization. That the dye was actually attached to the protein was verified by the fact that the pK of the dye changed by approximately 1 pH unit, and the scattering at pH = 3 was negligible in the protein solution.

Cotton was used as another support. The cotton was stained by boiling it for one hour in an aqueous 5% NaCl solution of mM Congo-red.⁵ After cooling, the cotton was removed, washed with running distilled water, and dried under vacuum. The stained cotton was suspended in D₂O at various pH values, and the resulting suspension was checked for NMR polarization. No polarization was observed. It appears from the foregoing that a solid state support is not sufficient for observation of the polarization, but some type of close aggregation between the Congo-red molecules is needed. Another possibility might be that the aggregation must be ionic in character.

Time Evolution of the NMR Signal. Figure 5A shows the time evolution of the polarization upon illumination of a freshly prepared solution, and Figure 5C shows the decay of the polarization upon removal of the light. The decay is exponential with a time constant of some 37 sec, a value reasonable for the proton T₁ of this system.⁶ It is independent of the illumination time. Figure 5B presents the time evolution of the polarization for a solution which had been pre-irradiated for some 40 minutes inside the NMR probe and then allowed to relax in the dark for several minutes to zero polarization.

The form of curve B in Figure 5 results from the diffusion of the polarized protons from the light guide-solution interface to the sensitive volume of the r-f coil. We have determined quantitatively the parameters of the diffusion equation. The details are omitted, as they serve no other purpose than to verify the origins of the curve.

The shape of curve A, Figure 5, is more difficult to rationalize since it is dependent upon sample history, the spinning rate, agitation after pre-irradiation, and temperature. The possibility of formation

of another chemical compound, which in turn is responsible for the polarization, was ruled out on the basis of arguments given above and the fact that samples which were pre-irradiated outside the probe and then reintroduced gave the same results as freshly prepared samples irradiated within the NMR probe. Additionally, the shape of type A curves depended on the sample spinning rate, as did the maximum polarization. The polarizations of a freshly prepared sample and a pre-irradiated sample were the same at 80°C.

Based on these observations, we infer that the difference between curves A and B is determined, mainly, by diffusion of the hydrosol particles toward the light guide interface; the conditions for maximizing the polarization are achieved when the particles form a thin layer beneath the light guide. In addition, the actinic light has a marked effect on the size of the hydrosol particles.

In sum, we believe that curve B shows the rise time for the steady state of polarized protons due to water diffusion, while curve A is a superposition of that phenomenon together with the rise time to achieve steady state in the macroscopic physical characteristics of the system.

Discussion

A few conclusions can be drawn from this work. The first is the finding that CIDNP signals could be observed in acidic suspensions of Congo-red. The observation of a net polarization is consistent with the fact that a radical pair, composed of two different radicals (different g values), was produced. The acidity of the solution was found not to be an essential factor in observing the polarization, but the physical characteristics of the solutions were found to be of primary

importance. Hydrosol formation was found to be essential for the observation of the polarization. The hydrosol state was formed either by working in acidic solutions or by salting out from neutral solutions. The NMR polarization was found to be proportional to the light intensity, which is indicative of formation of the polarization in the initial photochemical act. The polarized protons were observed in the solvent, indicating that they must be part of an exchangeable group. The only exchangeable protons in Congo-red (at least in the neutral form) are the amino group protons.

Scheme I is a suggestion of a mechanism which will account for all of these observations. The initial photochemical step proposed is the photoejection of a hydrogen atom from one of the amino groups of Congo-red (I). The resulting radical (II) forms a radical pair with the hydrogen atom. These two radicals can recombine to give back (I), but now, with polarized hydrogens on the amino group. The polarized protons will exchange with the solvent to cause the solvent polarization. The hydrogen atoms will easily escape from the cage and can finally recombine with radical (II) to produce another cage via direct or indirect processes. The final result of these processes is no net decomposition of the dye. We believe that these are the major events occurring, and they account for the very low quantum yield for decomposition. However, some of the hydrogen atoms may not reach (II) and may dissipate by recombination or by some other process. (II) in turn may disproportionate by reacting with its resonance form (III) to form (I) and (IV). (IV) has been identified as the major photochemical product of the photolysis of Congo-red.⁷ This mechanism can also account for the observation that the polarization could not be observed in solution. The reason is,

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Scheme I

probably, the very short lifetime of the resultant radical pair because of the very high mobility of the hydrogen atoms. This would also account for the observation that although most of the aromatic amines are known to photoeject hydrogen atoms⁸ and thus become photooxidized, no polarization has been observed for any of them.

The observation that upon cooling to 77°K radicals form only from the hydrosol suspension, cannot be explained solely in terms of reducing the macroscopic diffusion coefficients. It seems that a special microcrystalline arrangement is essential to block the back reaction. This microcrystalline arrangement is provided by the hydrosol formation, but not by cooling or by attaching the dye to a support like cotton wool, or a protein.

Conclusion. It was established in this work that CIDNP can be produced in macroscopic particles. The essential diffusional equilibrium is provided here by the relatively high mobility of the hydrogen atoms, while the observation of the polarization was made possible because of the fast exchange with the solvent.

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*This work was supported, in part, by the U.S. Atomic Energy Commission.

†N.I.G.M.S. Postdoctoral fellow.

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Figure Captions

Figure 1. Absorption spectra of Congo-red "solutions".

----- 3×10^{-5} M; pH = 5.
————— 3×10^{-5} M; pH = 2.5.
----- 10^{-4} M; the difference spectrum between irradiated and non-irradiated "solutions", both at pH = 2.5.

Figure 2. 60 MHz NMR spectra of H₂O in "solution" of 10^{-4} M Congo-red in ~99% D₂O and pD = 2.4. A: during irradiation. B: after irradiation.

Figure 3. Light intensity dependence of the NMR polarization for a "solution" of 10^{-4} M Congo-red in 99.8% D₂O at pD = 2.4.

Figure 4. EPR spectra of an irradiated "solution" of 10^{-4} M Congo-red in H₂O, pH = 2. The irradiation and the spectra were taken at 77°K. H· is the high field component of hydrogen atom signal created in the glass by irradiation.

Figure 5. Time evolution of the polarization of 10^{-4} M Congo-red in 99.8% D₂O, pD = 2.4. A: rise of the polarization of a fresh "solution" upon illumination. B: rise of the polarization of a pre-illuminated sample after equilibration in the dark to zero polarization. C: decay of the polarization following extinction of the light.
Note the different time scales for the upper and lower traces.

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Fig. 1

XBL 728-4724

Fig. 2

XBL 728-4734

Fig. 3

Fig. 4

XBL 728-4725

XBL728-4726

Fig. 5

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