For the most part the results have been considered to be more in line with the indirect, through-bond mechanism since many of the results appear inconsistent with the direct exchange mechanism. However, it is possible that both exchange mechanisms are of importance and together lead to the rather complex dependence of J on structure, solvent, and temperature.

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

References and Notes

(1) I, II, III, and IV were prepared from commercially available steroid diketones. 5α-*D*-homoandrostane-3,17-dione was prepared by the procedure of M. W. Goldberg et al., *Helv. Chim. Acta*, **23**, 376 (1940), and 5α-androstane-3,16-dione by the procedure of J. E. Bridgeman et al., *J. Chem. Soc. C*, 244 (1970). In general the diketone was refluxed with a 20-fold excess of 2-amino-2-methyl-1-propanol in xylene for 3 weeks as conversion to the D-ring oxazolidine was slow.
(2) J. F. W. Keana, S. B. Keana, and D. Beetham, *J. Am. Chem. Soc.* **89**, 3055 (1967).
(3) S. H. Glarum and J. H. Marshall, *J. Chem. Phys.*, **47**, 1374 (1967).
(4) High microwave power (100–250 mW) aids the detection of *S* resonances.
(5) For a complete list of all observed exchange energies, see E. K. Metzner, Ph.D. Thesis, University of California, Berkeley, 1974.
(6) The reported compounds all had satisfactory elemental analysis and mass spectral data.
(7) P. Michon and A. Rassat, *J. Org. Chem.*, **39**, 2121 (1974).
(8) F. V. Brutcher, Jr., and W. Bauer, Jr., *J. Am. Chem. Soc.*, **84**, 2236 (1962).
(9) N. L. Allinger and M. A. DaRooge, *J. Am. Chem. Soc.*, **83**, 4256 (1961).
(10) G. R. Underwood and H. S. Friedman, *J. Am. Chem. Spc.*, **96**, 4089 (1974).
(11) H. M. McConnell, *J. Chem. Phys.*, **33**, 115 (1960).
(12) E. K. Metzner, L. J. Libertini, and M. Calvin, *J. Am. Chem. Soc.*, **96**, 6515 (1974).
(13) B. R. Knauer and J. J. Napier, *J. Am. Chem. Soc.*, **98**, 4395 (1976).
(14) Hexane (20–60 °C), xylene (20–140 °C), chloroform (−60–60 °C).

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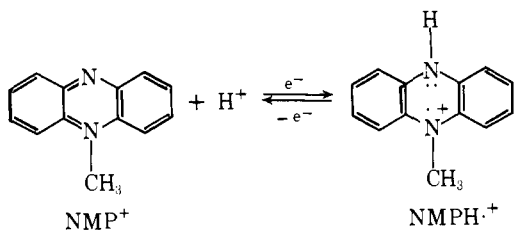
Nuclear Magnetic and Electron Spin Resonance Evidence for the Strength and Site of Attachment of *N*-Methylphenazonium Cation Radical to Sodium Dodecyl Sulfate Micelles[1]

Sir:

N-Methylphenazonium (NMP^+) cation salts[2] have been shown to be highly efficient promoters of cyclic photophosphorylation in photosynthetic systems.[3] Ample evidence exists to show that artificial cofactors, such as NMP^+, which are capable of stimulating the production of adenosine triphosphate (ATP) are lipophilic[4] and are able to translocate protons across a membrane.[5] The *N*-methylphenazonium cation radical ($NMPH^{+}\cdot$), obtained by one-electron reduction of NMP^+ (reaction 1), is in principle capable of carrying a proton across



(1)

a membrane since, on oxidation of $NMPH^{+}\cdot$, a proton is released. An a priori objection to involvement of $NMPH^{+}\cdot$ in photophosphorylation is that as a cation it would be expected to be hydrophilic and should therefore not readily interact with the hydrophobic part of the membrane. In an effort to understand the mechanism by which NMP^+ stimulates cyclic photophosphorylation we have now investigated the interaction of $NMPH^{+}\cdot$ with sodium dodecyl sulfate (NaLS) micelles. We have chosen micelles as perhaps the simplest model for the membrane-water interface. In this communication we report ESR and NMR results which bear on the strength of the interaction and the site of attachment of $NMPH^{+}\cdot$ to the NaLS micelle.

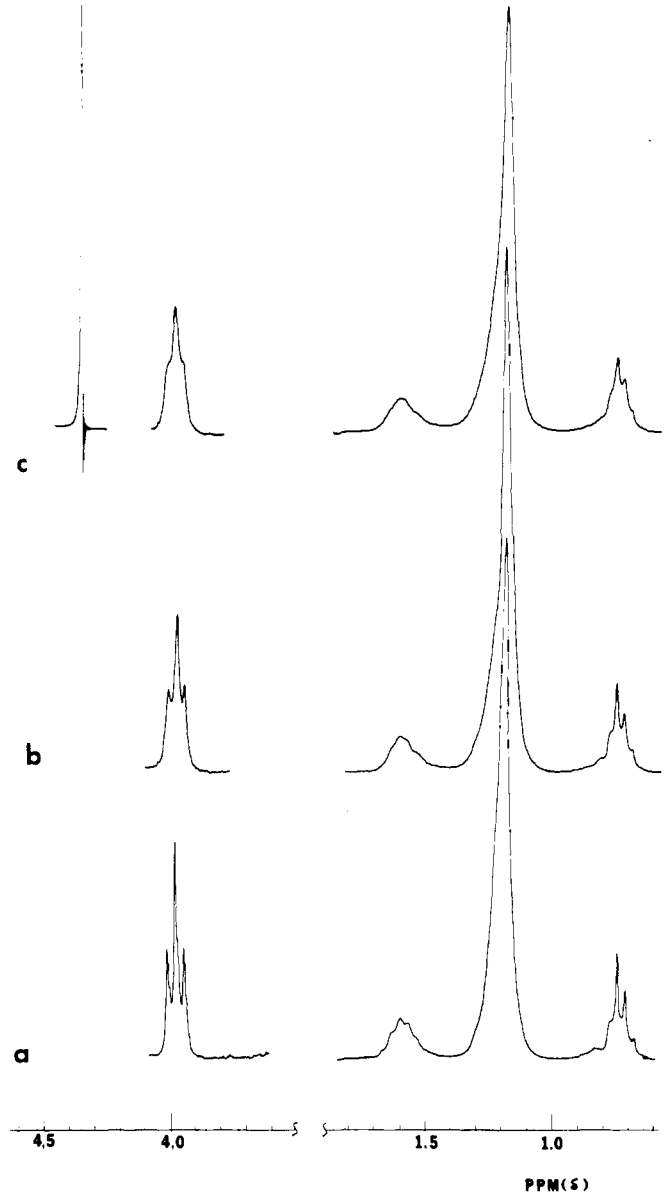


Figure 1. The 220-MHz NMR spectrum (Varian model HR220) of 0.1 M NaLS solutions in D_2O containing 10^{-2} M NMP^+ under conditions of varying photolysis time. There is no change in the HDO resonance during the course of these experiments and it is accordingly shown only once. The chemical shift indicated is in parts per million from TMS (external standard). NMR conditions for all spectra: sweep time 500 s, sweep width 1000 Hz, receiver gain 30 db, rf field level 20 db, signal amplitude 8.0 (for α-CH_2) and 3.2 (other resonances), frequency response 2 Hz. Photolysis time: (a) 0 s, (b) 5 s, (c) >3 min.

The ESR spectrum of $NMPH^{+}\cdot$ in water (produced either by visible light photolysis or sodium borohydride reduction of solutions of NMP^+ is rich in hyperfine structure, consisting of 5832 theoretical lines, over one hundred of which are resolved.[6-8] In contrast, the ESR spectrum of $NMPH^{+}\cdot$ in 0.1 M NaLS shows nine broad lines in which the small hyperfine

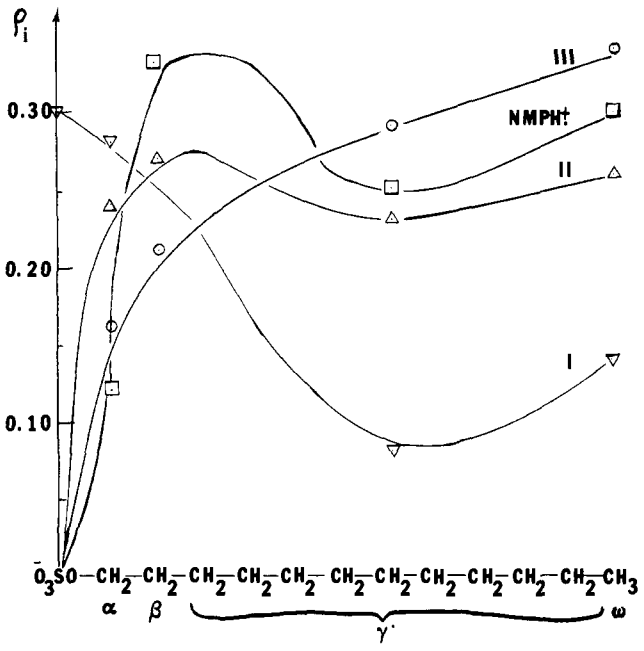


Figure 2. The x-axis is shown as the dodecyl sulfate anion with the resolvable proton resonances indicated just below the axis. The y axis is the normalized broadening ρ_i (= $\Delta\omega_i/\Sigma\Delta\omega_i$) where $\Delta\omega_i$ is the broadening observed for particular resolvable proton resonances of the dodecyl chain. Broadening for the sulfate head group is identified with the broadening observed for HDO. The following symbols were used for the various paramagnetic species: (a) $Mn(H_2O)_6^{2+}$ (compound **I**), ▽; (b) nitroxide **II**, △; (c) nitroxide **III**, ○; (d) $NMPH^{+}\cdot$, □.

splittings are not resolved.[9] This latter spectrum is indicative of a reduced isotropic tumbling rate for the $NMPH^{+}\cdot$ molecule. Since this broadened spectrum appears at concentrations of NaLS close to the published critical micelle concentration (CMC) of NaLS it appears that the phenomenon is concomitant with micellization. A strong binding of $NMPH^{+}\cdot$ to NaLS micelles is indicated with a binding constant (equilibrium constant for reaction 2), $K \simeq 10^5\ M^{-1}$.

$$NMPH_w^{+}\cdot + S_n \overset{K}{\rightleftharpoons} NMPH_m^{+}\cdot \qquad (2)$$

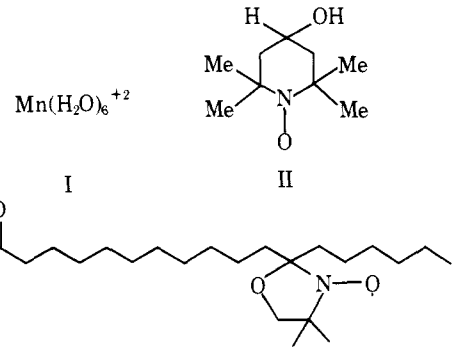
In reaction 2, $NMPH_w^{+}\cdot$ and $NMPH_m^{+}\cdot$ refer to aqueous and micellar $NMPH^{+}\cdot$, respectively, and S_n refers to the NaLS micelle. The ESR spectrum observed for $NMPH_m^{+}\cdot$ is very similar to that observed for binding of $NMPH^{+}\cdot$ to *Rhodospirillum rubrum* chromatophores[7] and to DNA.[8]

The results described above indicate a strong interaction between $NMPH^{+}\cdot$ and NaLS micelles. We now turn our attention to determining the site of attachment of $NMPH^{+}\cdot$ to the NaLS micelle. To do this we have taken advantage of paramagnetic broadening of proton NMR resonances of NaLS.

Theory predicts that the amount of broadening, $\Delta\omega_{1/2}$, experienced by a proton falls off as the inverse sixth power of the distance between the paramagnetic species and the proton,[10] i.e., $\Delta\omega_{1/2} \propto r^{-6}$. Hence protons which, on the average, are closer to a paramagnetic species will be broadened more than those which are farther away.

The 220-MHz NMR spectrum of NaLS is shown in Figure 1a. Resonances are readily assigned to α, β, γ' and ω protons. γ' refers to the unresolved methylene protons of the dodecyl chain (18 protons) and ω refers to the terminal methyl group. If NMP^{+} is added and the sample is briefly photolyzed to produce some $NMPH^{+}\cdot$, the proton NMR resonances of NaLS are observed to broaden (Figure 1b). Continued photolysis leads to increased broadening until the $NMPH^{+}\cdot$ concentration reaches its maximum value[11] (Figure 1c). Plots of the broadening of NaLS protons as a function of photolysis time (i.e., increasing $NMPH^{+}\cdot$ concentration) show that the largest increase in broadening is observed for the β position, the next largest at the ω position, the next at the γ' position, and the smallest at the α position. No change in the line width of the HDO resonance is seen over the range of these experiments. The implication of these results is that $NMPH^{+}\cdot$ is, on the average, closer to protons down the chain than it is to those protons close to the sulfate head groups. In terms of micelle structure, this would imply that $NMPH^{+}\cdot$ spends much of its time in the hydrophobic region of the micelle.

To test this possibility we have made measurements of NaLS solutions of stable paramagnetic species where the relative hydrophobicity-hydrophilicity of the paramagnetic species could be gauged in advance. The systems we have examined in this manner are hexaaquomanganous ion (**I**) and the nitroxides II and III. We chose I as an example of a hydrophilic



paramagnet, III as a hydrophobic species, and II in the hope that it would behave as a radical of intermediate hydrophobicity. The results of these experiments are summarized in Figure 2, along with those described above for $NMPH^{+}\cdot$.[13]

From these experiments it appears that this technique of using paramagnetic broadening of NMR resonances is useful for locating paramagnetic species in micelles. That is, the hydrophilic manganous ion causes the greatest broadening in the surface region, the hydrophobic nitroxide, III, has its greatest effect in the hydrocarbon center, and the nitroxide of intermediate hydrophobicity, II, is most effective somewhere between the polarity extremes of the NaLS micelles. The results for this latter case most closely resemble those obtained for the $NMPH^{+}\cdot$ molecule.

Careful examination of the optical absorption spectrum of NMP^{+} as a function of NaLS concentration reveals that NMP^{+} also interacts strongly with the NaLS micelle. However, this interaction occurs in a region that is very hydrophilic, probably at the water-micelle interface.[14] This is consistent with the probable site of reaction of NMP^{+} with the photosynthetic electron transport chain.[5]

The experiments described in this communication lead us to conclude that $NMPH^{+}\cdot$ binds strongly to NaLS micelles. The site of attachment of $NMPH^{+}\cdot$ to the micelle is in the hydrocarbon region a few angstroms below the head groups. This latter result is consistent with the observed enhancement of the rate of cyclic photophosphorylation by NMP^{+} which would require a strong interaction of a reduced form of NMP^{+} with the membrane. Moreover, our results indicate that the actual proton transport agent may well be $NMPH^{+}\cdot$ rather than the doubly reduced NMPH as had been previously suggested.[4a,15]

Acknowledgment. It is a pleasure to acknowledge the Ontario Research Foundation for the use of their Varian 220-MHz NMR instrument. We acknowledge helpful comments and criticisms from Professor Julien Genyea.

References and Notes

(1) This research was supported by a grant from the National Research Council of Canada and by Research Contract No. 1SQ5-0111 from Supply and Service Canada.
(2) The most common salt is *N*-methylphenazonium methylsulfate which is often referred to in the literature as PMS. We have chosen not to use this abbreviation because the phenomena we examine in this paper are independent of the anion. The abbreviation NMP^+ is specific to the cation.
(3) A. T. Jagendorf and M. Avron, *J. Biol. Chem.*, **231**, 277 (1958).
(4) (a) G. Hauska, *FEBS Letters*, **28**, 217 (1972); (b) G. Hauska, A. Trebst, and W. Draber, *Biochim. Biophys. Acta.*, **305**, 632 (1973); (c) G. Hauska and R. C. Prince, *FEBS Letters*, **41**, 35 (1974).
(5) A brief review is given by A. Trebst in *Proc. Int. Congr. Photosynth., 3rd*, I, 439–448 (Sept. 2–6, 1974). Elsevier Scientific Publishing Company, Amsterdam, The Netherlands, 1974.
(6) V. S. F. Chew, Ph.D. thesis, University of Western Ontario, London, Canada, 1977.
(7) K. Cost, J. R. Bolton and A. W. Frenkel, *Proc. Natl. Acad. Sci.*, **57**, 868 (1967).
(8) K. Ishizu, H. H. Dearman, M. T. Huang, and J. R. White, *Biochemistry*, **8**, 1238 (1969).
(9) This ESR spectrum is clearly due to the $NMPH^{+}\cdot$ cation radical and not the neutral $NMP\cdot$ radical. The gross hyperfine pattern can be analyzed in terms of two nearly equivalent nitrogens and four nearly equivalent protons (the three methyl protons and one N-H proton). By analogy with protonated and methylated nitrogens in other nitrogen heterocyclic radicals we expect that $a^N \sim a^H$. If the equivalences were exact then a nine-line pattern with intensities 1:6:17:30:36:30:17:6:1 would be expected which is close to the intensity ratios of the observed nine-line pattern. Further confirmation is obtained from the ESR spectrum in D_2O where the hyperfine pattern collapses to an eight-line spectrum as expected for one exchangeable hydrogen.
(10) A. Carrington and A. D. McLachlan, "Introduction to Magnetic Resonance", Harper and Row, New York, N.Y., 1967, p 228.
(11) Disproportionation of $NMPH^{+}\cdot$ limits its concentration[12] according to $2NMPH^{+}\cdot \rightleftharpoons NMP^{+} + NMPH + H^{+}$. Under our conditions (pH $\simeq$7, $[NMP^+] \simeq 10^{-3}$ M), $[NMPH^{+}\cdot]_{max} \simeq 0.2\,[NMP^+]_0$.
(12) W. S. Zaugg, *J. Biol. Chem.*, **239**, 3964 (1964).
(13) The interpretation of these experiments rests on the assumption that we can consider these paramagnetic probes as point dipoles. Clearly they do have a finite size; however, even the $NMPH^{+}\cdot$ is small compared to the $\sim$ 40-Å diameter of the micelle.
(14) C. A. Evans and J. R. Bolton, unpublished results.
(15) Of course, if NMPH were to be the proton translocating species, a two-electron oxidation would be necessary for a proton to be liberated. This would be only half as efficient for proton transfer as the scheme proposed in this paper.
(16) Contribution No. 168 from the Photochemistry Unit.

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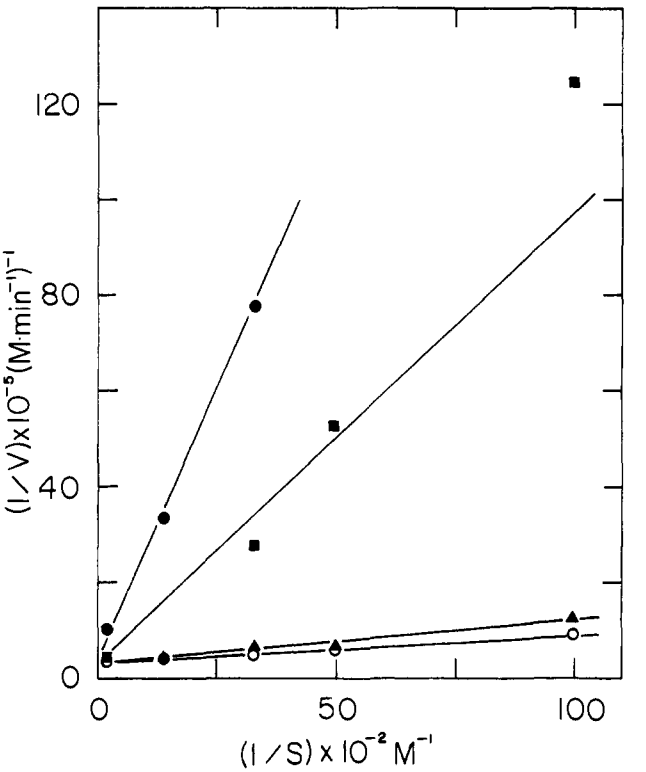
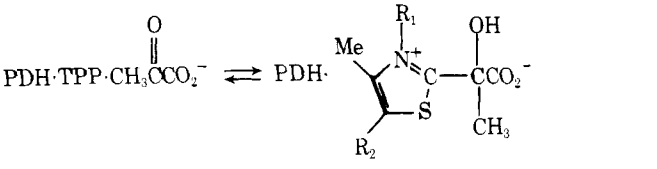


Figure 1. Lineweaver–Burke plot of the initial rate of conversion of pyruvate S to acetyl coenzyme A, catalyzed by *E. coli* PDH (3×10^{-3} units/mL) at 25 °C, pH 7.65 (0.05 M phosphate buffer) in the presence of MAP (concentrations (μm): ●, 1.5; ■, 0.6; ▲, 0.1; ○, 0.0). TPP (200 μM), coenzyme A (60 μM), NAD^+ (2 mM), and magnesium chloride (1 mM), according to the procedure of Reed and Mukherjee.[20] The appearance of NADH was followed at 340 nm with a Hitachi–Coleman recording spectrophotometer. The value of K_i, determined by a replot of the slopes of the lines in the figure (method in ref 21), is 5×10^{-8} M.



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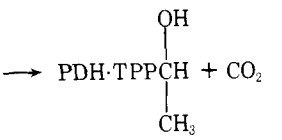

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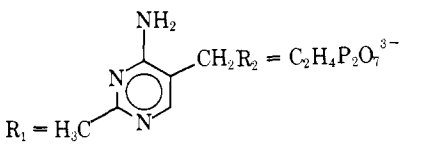

Active Site Generated Analogues of Reactive Intermediates in Enzymic Reactions. Potent Inhibition of Pyruvate Dehydrogenase by a Phosphonate Analogue of Pyruvate[1]

Sir:

Pyruvate dehydrogenases (PDH) are multienzyme complexes responsible for the conversion of pyruvate to acetyl coenzyme A.[2] The initial steps are catalyzed by a component enzyme (E_1) which promotes the decarboxylation of pyruvate. This requires the coenzyme, thiamine pyrophosphate (TPP).[3] A detailed mechanism has been proposed[4,5] that is based on an analogy to the mechanism proposed for condensation reactions which are catalyzed by thiazolines (eq 1).

This mechanism requires the initial conversion of pyruvate to an enzyme-bound, covalent adduct of TPP, 2-lactyl TPP (**I**).[6] The intermediate is converted to an isolable product, hydroxyethyl TPP (HETPP). The involvement of I and the mechanism of its decarboxylation are of considerable interest but the properties of compounds related to I weigh against the isolation of I from an enzymic reaction.[7]

A promising method for experimental confirmation of the involvement of specific covalent intermediates has been developed by Westerik and Wolfenden[8,9] and by Thompson.[10] This is a modification of the use of transition state analogues.[11–13] The method is exemplified by the powerful inhibition of papain[8] and of elastase[10] by aldehydic analogues of peptide substrates. Peptides are normally cleaved in a catalytic step following addition of an enzymic hydroxyl or sulfhydryl group to the carbonyl moiety of the peptide via a supposed tetrahedral intermediate (T). However, it was proposed that the aldehyde forms readily and reversibly a hemiacetal or thiohemiacetal (T′) which may only revert to the starting species. Since the enzyme's catalytic function involves stabilization of normally reactive species, the powerful inhibition by aldehydes supports the existence of the proposed intermediate (T).