[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, FACULDADE DE FILOSOFIA, CIENCIAS E LETRAS DA UNIVERSIDADE DE SÃO PAULO]

The Polarity of a Model for Reduced Pyridine Nucleotides

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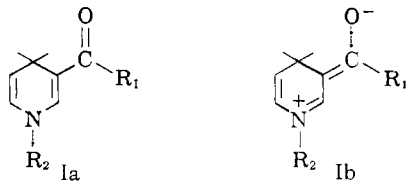
In 1-benzyldihydronicotinamide, the contribution of the dipolar structure, as indicated by dipole moment and spectral data, although important, is not exceptionally large. The study is extended as to explain the spectral differences between the 1,4- and 1,6-dihydronicotinamides.

Usually structure Ia ($R_1 = NH_2$; R_2 = ribose–pyrophosphate–adenosine) is assigned to reduced diphosphopyridine nucleotide (hereafter called DPNH). Kaplan and Ciotti[1] in order to explain the batho- and hyperchromic shifts on passing from DPNH to its 3-acetyl analog (I, $R_1 = CH_3$; R_2 = ribose–pyrophosphate–adenosine) have suggested that the reduced coenzyme and the analog could exist in the dipolar structure Ib. They also considered it possible that the conjugated form Ib is the stable form in which DPNH exists.



Ia Ib

Structure Ib as a resonating form of DPNH has also been implicated in the mechanism of action of the yeast alcohol dehydrogenase.[2,3]

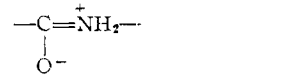
It was therefore of interest to investigate the actual contribution of the ionic structure Ib to I; this has been done by ascertaining the dipole moment as well as the absorption spectrum in solvents of different polarities of a model for DPNH, 1-benzyldihydronicotinamide[4] (I, $R_1 = NH_2$; $R_2 = -CH_2-C_6H_5$).

Initially it may be said that an important contribution of the dipolar structure is to be expected in analogy with certain β-amino-α,β-unsaturated ketones[5] and indeed 1,4-dihydronicotinamides do not parallel the basicity of Δ^2-tetrahydropyridines (where a simple derivative, the 1,2-dimethyl compound, has a pK_a of 11.43[6]).

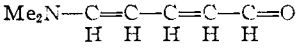
It is to be noted that for a substantial contribution of the charged structure the geometry of the dihydropyridine ring should change from the boat configuration toward a planar configuration. Indeed, for effective resonance in the vinylogous amide group, trigonal hybridization at the ring nitrogen is necessary, for then the unshared pair of electrons will have π-symmetry. As a consequence, all the other ring atoms will lie in the same plane except the C_5 atom, if the tetrahedral angle at C_4 is to be maintained.

Compound I is a merocyanine and its spectral behavior examined as such[7] indicates that, to a first approximation, the structure can be interpreted in terms of Ia ⟷ Ib resonance, with the uncharged structure contributing much more than the charged one.

Thus, the highest value of λ_{max} for the longer transition in the spectrum of the model coenzyme is found in water (357 mμ). In other solvents the position is as follows: chloroform (354 mμ), ethyl alcohol (354 mμ), benzene (349 mμ), acetone (347 mμ), dioxane (345 mμ), cyclohexane (345 mμ) and ethyl ether (340 mμ). This means that water stabilizes the dipolar structure; the dissimilarity of the extreme structures is reduced, the interaction increased, yet the transition energy reduced. The same result can be more effectively obtained by stabilizing Ib through loss of the competing $-CONH_2$ resonance: DPNH absorbs at 340 mμ, yet the 3-acetyl analog has its maximum at 365 mμ. This large bathochromic shift indicates that the competition of the amidic resonance is strong. Structure



should even be more important than structure Ib, since the latter is hampered by the cross-conjugated vinylamine resonance. Hence the contribution of the dipolar structure Ib to I is markedly reduced and this explains why the effect of solvent polarity upon λ_{max} is quite small. As a comparison, it may be reported that λ_{max} of



is shifted 40 mμ toward shorter wave lengths on passing from aqueous solution to a methanol solution.[8]

It is interesting to note in passing that the suggestion of Kaplan and Ciotti,[9] according to which the hypsochromic shift observed when DPNH is bound to liver alcohol dehydrogenase is due to a hydrogen bond between a group of the protein and the pyridine nitrogen, is supported by the present views, for such a bond will stabilize the uncharged structure and will therefore increase the dissimilarity between Ia and Ib.

The dipole moment of 1-benzyldihydronicotinamide in dioxane solution is 3.89 *D*. As an approximation we consider the reduced model a diamide and note that the electric moment is between that

(1) N. O. Kaplan and M. M. Ciotti, *J. Biol. Chem.*, **221**, 823 (1956).

(2) H. R. Mahler and Joyce Douglas, THIS JOURNAL, **79**, 1159 (1957).

(3) K. Wallenfels and H. Sund, *Biochem. Z.*, **329**, 59 (1957).

(4) D. Mauzerall and F. H. Westheimer, THIS JOURNAL, **77**, 2261 (1955).

(5) See for instance N. H. Cromwell, F. A. Miller, A. R. Johnson, R. L. Frank and D. L. Wallace, *ibid.*, **71**, 3337 (1949).

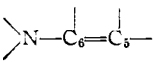
(6) R. Adams and J. E. Mahan, *ibid.*, **64**, 2588 (1942).

(7) L. G. S. Brooker, *et al.*, *ibid.*, **73**, 5332 (1951).

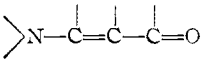
(8) D. L. Peterson and W. T. Simpson, *ibid.*, **79**, 2375 (1957). The authors are grateful to a referee for calling attention to this point.

(9) N. O. Kaplan and M. M. Ciotti, *J. Biol. Chem.*, **211**, 431 (1954).

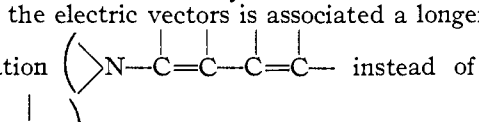
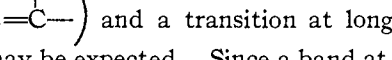
of formamide (3.0 *D*)[10] and that of urea (4.56 *D*)[11]; this we ascribe to opposition, in the coenzyme model, by the moment of the benzyl group and by the moment of the

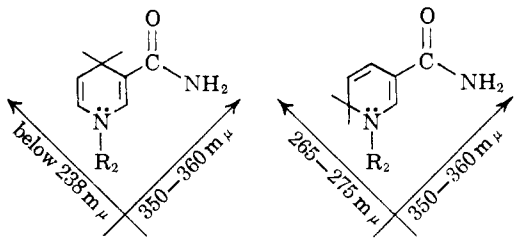


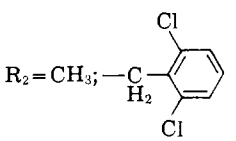
resonance. The energetic dissimilarity between the two structures having a positively charged nitrogen is also a reason for the fact that the moment of the model is lower than that of urea. At any rate, it can be deduced that the resonance moment of the



grouping cannot be exceptionally large and that DPNH does not have simply structure Ib.

Since in I the vinylamine group and the vinylogous amide group are cross-conjugated with electric moments approximately at right angle to each other, it may be possible to excite by light these two groups independently of each other.[12] Such a vinylamine band would be expected below 238 mμ,[13] but in 1,6-dihydronicotinamides with one of the electric vectors is associated a longer conjugation  instead of  and a transition at longer wave length may be expected. Since a band at 265–275 mμ appears in the spectrum of certain dihydronicotinamides[14–18] we take this fact as a further indication of 1,6-dihydro structure.[19]





Finally, a few remarks on the absorption intensities may be in order. It may be expected that in a molecule such as I (R = NH_2) removing the cross-conjugation at whatever end of the chromophoric group there is an increase of the absorption intensity. Indeed, on passing from DPNH to its acetyl analog the area of the longer wave length increases.[20] From work of Wallenfels and Schuly[21] it can be inferred that the longer transition is much stronger in 1,4,5,6-tetrahydronicotinamides than in the dihydronicotinamides. Comparison between the isomeric dihydronicotinamides is at present unwarranted in view of the scanty data.[22]

Experimental

Preparation of 1-Benzyldihydronicotinamide.—Nicotinamide 1-benzylochloride (m.p. 234°) was prepared according to Karrer and Stare[23] from nicotinamide and benzyl chloride and in turn reduced in alkaline solution with Merck sodium dithionite to 1-benzyldihydronicotinamide.[4,23] The compound was recrystallized several times from ethanol-water and dried *in vacuo*. The product melted with decomposition; when the heating was as short as possible with equilibrium conditions assured, the range of melting was reduced to 4° and the melting point was 121°.

Ultraviolet Absorption Spectra of 1-Benzyldihydronicotinamide.—Measurements were taken with a Beckman D.U. spectrophotometer employing solutions in purified solvents. The readings were extended as far as possible to the side of shorter wave lengths. Cyclohexane, ethyl ether and probably chloroform solutions show also a shoulder in the 240 mμ region. Several of the solutions in solvents of low dielectric constant became turbid after a period of time, short in the case of carbon tetrachloride, longer with cyclohexane, benzene and ethyl ether.

Purification of Dioxane for Dipole Moment Measurements.—A first purification was carried out according to Vogel[24]; it was then fractionally crystallized and fractionally distilled over sodium (n_D 1.4200; ϵ_{25} 2.209; b.p. 98.5° (695.6 mm.)). The degree of purity, Δt, in the Swietoslawski[25] ebulliometer was 0.00 ± 0.02. It was stored over sodium.

TABLE I

ω_2	ϵ_{12}	ν_{12}
0.008419	2.289	0.97129
.011430	...	.97081
.012292	2.324	.....
.015342	...	.97017
.016556	2.363	.....
.019262	2.388	.96950

ϵ_1	ν_1	α	$-\beta$	P_{20}	R_D	μ
2.211	0.97266	9.154	0.166	372.5	63.15	3.89

Density measurements were made at 25.00 ± 0.03° as reported in earlier work from these laboratories.[26]

Dielectric constant measurements were carried out at 25.00 ± 0.02° in an apparatus of the heterodyne-beat type to be reported in details later.[27] Pertinent data are collected in Table I. The molar polarization of the solute at infinite dilution P_{20} was calculated by the method of Halverstadt and Kumler.[28] The distortion polarization was taken equal

(10) G. D. Burdun and P. B. Kantor, *Doklady Akad. Nauk S.S.S.R.*, **67**, 985 (1949).

(11) W. D. Kumler and G. M. Fohlen, THIS JOURNAL, **64**, 1944 (1942).

(12) See for instance H. P. Koch, *J. Chem. Soc.*, 387 (1949).

(13) *Cf.* R. Tschesche and G. Snatzke, *Ber.*, **90**, 579 (1957).

(14) G. Stein and A. J. Swallow, *Nature*, **173**, 937 (1954).

(15) G. Stein and G. Stiassny, *ibid.*, **176**, 734 (1955).

(16) G. Stein and A. J. Swallow, *J. Chem. Soc.*, 306 (1958).

(17) K. Wallenfels and H. Schuly, *Ang. Chem.*, **67**, 517 (1955).

(18) K. Wallenfels and H. Schuly, *ibid.*, **69**, 505 (1957).

(19) K. Wallenfels and H. Schuly, *ibid.*, **69**, 139 (1957).

(20) See Fig. 3A of ref. 1.

(21) K. Wallenfels and H. Schuly, *Biochem. Z.*, **329**, 75 (1957).

(22) The ϵ_{max} for 1-methyldihydronicotinamide presumed to be 1,6-tautomer is 3500, as calculated from data published by Stein and Swallow.[14] For the 1,4-tautomer the figure is 7000 (G. W. Rafter and S. P. Colowick, *J. Biol. Chem.*, **209**, 773 (1954)). The ϵ_{max} for enzymatically inactive DPNH obtained by electrolytic reduction at the Hg electrode (B. Ke, *Arch. Biochem. Biophys.*, **60**, 505 (1956)) can be estimated as being 4000, and for enzymatically inactive DPNH obtained by sodium borohydride reduction (M. O. Mathews and E. E. Conn, THIS JOURNAL, **75**, 5428 (1953)) a value of 3200 has been deduced. Enzymatically active DPNH has a value of 6220 (B. L. Horecker and A. Kornberg, *J. Biol. Chem.*, **175**, 385 (1948)).

(23) P. Karrer and F. J. Stare, *Helv. Chim. Acta*, **20**, 418 (1937).

(24) A. I. Vogel, "A Text-book of Practical Organic Chemistry," Longmans, Green & Co., New York, N. Y., 2nd ed., 1951, p. 175.

(25) W. Swietoslawski, "Ebulliometric Measurements," Reinhold Publ. Corp., New York, N. Y., 1945.

(26) S. Mathias, THIS JOURNAL, **72**, 1897 (1950).

(27) S. Mathias and E. de Carvalho Filho, unpublished.

(28) I. F. Halverstadt and W. D. Kumler, THIS JOURNAL, **64**, 2988 (1942).

to the molar refraction R_D as calculated from bond refractions values.[29]

(29) A. I. Vogel, W. I. Creswell, G. H. Jeffery and J. Leicester, *J. Chem. Soc.*, 514 (1952).

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Dielectric Properties of Hemoglobin. VI. Measurements with Solid Materials

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The dielectric increments and the dispersions of horse oxy- and carboxyhemoglobin in the frozen state were measured between −5 and −40° in a frequency range of 400 kc. to 18 mc. A considerable decrease of the dielectric constants of both hemoglobins were observed on freezing the solution. The dielectric increments per gram at −5° are 0.10 and 0.02 for oxy- and carboxyhemoglobin, respectively. The dielectric constant of oxyhemoglobin crystals was measured in the same temperature and frequency range. Analysis of the anomalous dispersion and its temperature dependence was based on the results obtained with the crystal sample. It was found that the dielectric polarization of oxyhemoglobin passes through at least three consecutive stages with decreasing temperature. The dipole moments obtained were in good agreement with the theoretical value of Kirkwood.

Introduction

The dielectric polarization of protein molecules has been explained by the orientation of the whole molecule in the electric field. Thus, Oncley's[1] results on the anomalous dispersion of hemoglobin solutions were successfully interpreted by means of the rotary diffusion theory. However, recently Jacobsen[2−4] suggested that orientation of the whole molecule is not likely to occur in the dielectric polarization of protein solutions and that excitation of the hydrated water molecules is the substantial mechanism of the polarization. Similarly, Bayley[5] stated that the polarization of protein crystals increases in the presence of sorbed water. On the basis of dipole moment calculations, Kirkwood[6] suggested that the induced moment, which he attributed to fluctuation of mobile protons, is the major part of the dipole moment of some protein molecules, including hemoglobin.

The experimental approach to this problem is difficult. A rather indirect experiment of Timasheff, *et al.*,[7] on the charge fluctuation of albumin by the light-scattering method indicates that the dipole interaction of albumin molecules accompanies the fluctuation of 3.58 protonic units per molecule.

Therefore, we have studied wet hemoglobin crystals and also frozen samples in order to observe the polarizability in the presence of sorbed water. The results have been analyzed thermodynamically and discussed in terms of the dielectric theories concerning protein molecules.

Experimental

The bridge substitution method was used for measurement of the dielectric constants. Since the capacity and conductivity of ice are very small in the radiofrequency range, the Twin T bridge which is suitable for the measurement of the large impedance, was used. The frequency in this experiment is high enough to be outside the dispersion region of ice. Therefore, it behaved like a nonpolar substance and had a dielectric constant of about 4 which is favorable for the observation of the dielectric increment. When water is used as the solvent, the small increase of dielectric constant due to the protein molecules is difficult to detect because of the high conductivity and large dielectric constant of water itself. However, since in our case the dielectric increment has the same order of magnitude as the dielectric constant of the ice, the measurements are very precise. The dielectric constant of ice is almost independent of the temperature at these frequencies and should have a constant value throughout the radio-frequency range. However, the bridge does not function properly at high frequency and a slight decrease of capacity was always observed above 10 megacycles. The dielectric increment of protein at each frequency was obtained by subtracting the dielectric constant of ice at the same frequency from the total dielectric constant. Thus the total dielectric increment of the protein solid is given by the following equation; $\Delta\epsilon_t = (\epsilon_t - \epsilon_i)_0 - (\epsilon_t - \epsilon_i)_\infty$ where ϵ_t and ϵ_i are the total dielectric constant and the dielectric constant of ice, respectively, and $(\epsilon_t - \epsilon_i)_0$ and $(\epsilon_t - \epsilon_i)_\infty$ are the dielectric constant of protein at low and at high frequencies.

Because of the very small dielectric constant of the material, the cell design and the effect of connecting leads are important. Precautions were taken to minimize the effect of stray capacity and an electrode distance was selected within the region in which the capacity bears a linear relation to the electrode distance. The electrodes were plated with platinum black and practically no electrode polarization was observed in this frequency range. The electrodes were completely enclosed in the frozen material.

The temperature was varied from −4 to −40°. The protein solution (15 to 25 g. per liter) was frozen quickly by immersion of the solution cell in Dry Ice–acetone. The capacity readings were repeated until a constant value was obtained. The temperature control was far from ideal. Since the measurement over the entire frequency range takes considerable time, some heat exchange was unavoidable even though the cell was enclosed in a Dewar flask.

Results

The dielectric constants of frozen horse oxyhemoglobin are shown in Fig. 1, in which $\epsilon_t - \epsilon_i$ is plotted against the logarithm of the frequency in mc. It is obvious that the protein solid shows anomalous dispersion in nearly the same frequency range as the solution. The temperature dependence of the dielectric increments and of the relaxation time are plotted in Figs. 2 and 3,[8] respectively.

(1) J. L. Oncley, THIS JOURNAL, **60**, 1115 (1938).

(2) B. Jacobsen, *Rev. Sci. Instr.*, **24**, 10, 249 (1953).

(3) B. Jacobsen and M. Wenner, *Biochim. Biophys. Acta*, **13**, 577 (1954).

(4) B. Jacobsen, THIS JOURNAL, **77**, 2919 (1955).

(5) S. T. Bayley, *Trans. Faraday Soc.*, **47**, 5, 509 (1951).

(6) J. Kirkwood and J. B. Schumaker, *Proc. Nat. Acad. Sci.*, **38**, 855 (1952).

(7) S. N. Timasheff, H. H. Dintzis, J. Kirkwood and B. D. Coleman, THIS JOURNAL, **79**, 782 (1957).

(8) The values for the temperature above the freezing point are taken from the previous paper, to be published in *Arch. Biochem. Biophys.*