Nuclear Magnetic Resonance Study of the Conformation of Nicotinamide–Adenine Dinucleotide and Reduced Nicotinamide–Adenine Dinucleotide in Solution*

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ABSTRACT: The findings reported here are consistent with the conclusion that the pyridine nucleotides can exist in solution as an equilibrium mixture of folded and unfolded forms. For both nicotinamide–adenine dinucleotide and reduced nicotinamide–adenine dinucleotide, addition of methanol or urea to the neutral aqueous solution leads to an increase of the ratio of the unfolded to folded forms. In the case of nicotinamide–adenine dinucleotide precise chemical shift data obtained over a wide pH range agree with the data reported by Jardetzky and Wade-Jardetzky but disagree with the data reported by Sarma, Ross, and Kaplan. On the other hand for reduced nicotinamide–adenine dinucleotide we find that, in contrast to results reported by Jardetzky and Wade-Jardetzky, the C-2 and C-4 protons of the reduced pyridine ring show chemical shifts that are essentially independent of pH in the pH range 2–10.

Fluorescence transfer data for NADH suggest that there is an intramolecular interaction between the adenine and reduced pyridine rings (Weber, 1957; Velick, 1958; Freed *et al.*, 1967). Nuclear magnetic resonance studies proved particularly advantageous in demonstrating that this interaction occurs in NAD as well as in NADH, and that it involved a folding of the dinucleotides with the two nucleotide rings stacked nearly in parallel (Meyer *et al.*, 1962; Jardetzky and Wade-Jardetzky, 1966). In view of certain discrepancies in results reported by others (Jardetzky and Wade-Jardetzky, 1966; Sarma *et al.*, 1968), we have carefully reexamined the pH dependence of the nuclear magnetic resonance spectra of NAD and NADH. In addition, we were interested in the effect of environment on the folding of these dinucleotides. If, for example, NAD and NADH unfold when they are near hydrophobic regions of proteins, unfolding should also be observed in solvents that are less polar than water (Freed *et al.*, 1967). In this connection we have examined the effect of methanol and urea on the dinucleotide chemical shifts. This paper reports the results of these studies.

Experimental Procedure

All nuclear magnetic resonance spectra were obtained on a Varian HA-100 spectrometer operating in the frequency sweep mode. At the lower concentrations the Varian C-1024 computer was used to enhance the signal-to-noise ratio. The spectrometer was modified so that the frequency sweep was driven by the C-1024 through a voltage-controlled variable frequency oscillator. In single scan spectra chemical shifts were measured by stopping the spectrometer pen at the maximum of each response and counting for 10 sec the frequency difference from a capillary tube containing tetramethylsilane used as a lock signal. The position of the resonance of about 0.1% DSS[1] contained in each sample was also determined, and all shifts are reported relative to internal DSS. For multiple scan spectra the C-1024 sweep width was calibrated by direct counting, and resonance positions were determined by interpolation. The precision of the chemical shift measurements is ±0.01 ppm. NAD and NADH were obtained from Sigma and used without further purification. The samples were lyophilized from D_2O to remove exchangeable hydrogens and the nuclear magnetic resonance measurements were made immediately. In the pH studies, the pH was adjusted by additions of solutions of DCl or KOD in D_2O and the values reported are direct pH meter readings. The pH was rechecked after each measurement and only those with a constant pH were retained.

Results and Discussion

Figure 1 illustrates the pH[2] dependence of the chemical shifts for 0.05 M NAD at 32°. These results agree with those reported previously (Jardetzky and Wade-Jardetzky, 1966) in that the magnitude of the total shifts between pH 1 and 10 are about the same and in that a typical titration curve with a single pK is observed. The pH is 3.5 in Figure 1 and about 4 in the figure published by Jardetzky and Wade-Jardetzky. Conflicting data (Sarma *et al.*, 1968), indicating a

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[1] Abbreviation used is: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

[2] The pH arises from deuterium ions, but the values reported are those obtained directly from readings made with a pH meter. The corresponding pD values are 0.40 unit higher (Glasoe and Long, 1960).

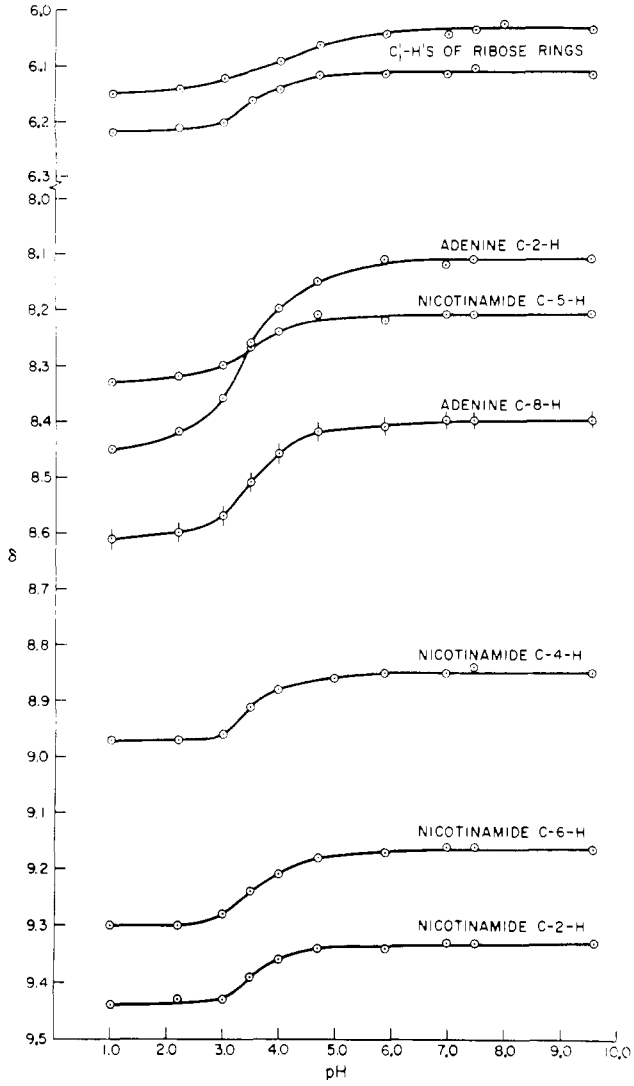

FIGURE 1: The effect of pH[1] on the chemical shifts for the indicated protons of NAD. The precision of the individual points in this figure and Figure 2 is illustrated by the bars associated with the data for the chemical shifts of the proton attached to C-8 of adenine.

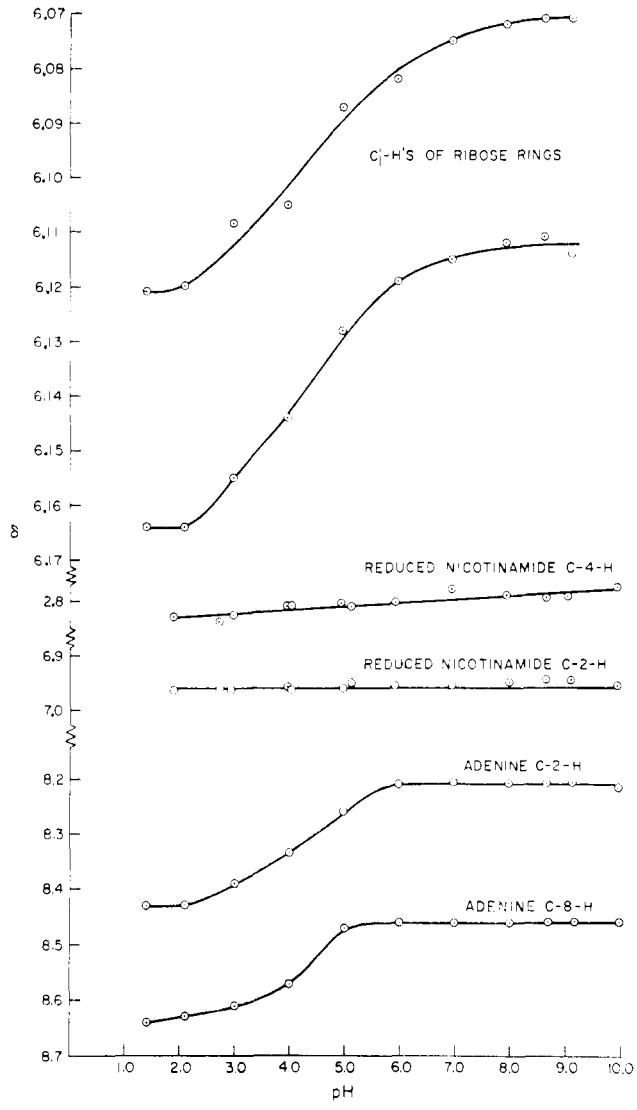

FIGURE 2: The effect of pH[1] on the chemical shifts for the indicated protons of NADH.

minimum chemical shift around pH 7 and interpreted as "a maximum intramolecular interaction in the biological pH range," evidently is in error. It was pointed out (Jardetzky and Wade-Jardetzky, 1966) that the observed transition between pH 3 and 4 almost certainly reflects the pK of the adenine N-1-H at 3.88. Since the titration curve for NAD show no pK between 5 and 10 (Moore and Underwood, 1969; Y. M. Lee and C. F. Walter, 1969, unpublished data), a minimum at pH 7 is not expected.

The pH dependence of the chemical shifts for a solution of 0.05 M NADH at 32° is illustrated in Figure 2. The adenine C-2 and C-8 proton resonances again move upfield in the pH range near the pK of the adenine N-1-H; the magnitudes and the pK which we observe for these adenine protons agree quite well with the Jardetzkys' results. However, in contrast to the results reported by these workers, we find that the chemical shifts for the C-2 and C-4 protons of the reduced nicotinamide ring are essentially independent of pH, although a slight pH dependence might be read into the data in the case of the C-4 proton. The H'$_1$ proton shifts also show little or no pH dependence. We were unable to obtain NADH spectra below pH 2 because the reduced nucleotide decomposed rapidly at lower pH.

In contrast to the situation in NAD, the reduced nicotinamide ring of NADH is not positively charged, and despite claims to the contrary (Kosower, 1962), it should not be protonated in the pH range above 2. Protonation in dilute acid does occur at C-5 of the reduced nicotinamide ring (Anderson and Berkelhammer, 1958; Kim and Chaykin, 1968), but this reaction is too slow to cause the ring to become positively charged during the experiments reported here. Thus, the protonation of the adenine ring between pH 3 and 4 is not expected to produce unfolding of NADH. The results with NADH reported in Figure 2 bear out this expectation. Thus, the folded conformation can continue to exist provided either (but not both) of the nucleotide rings is positively charged. The pH-dependent shifts of the adenine protons of NADH are apparently due almost entirely to the effect of protonation rather than unfolding of the molecule.

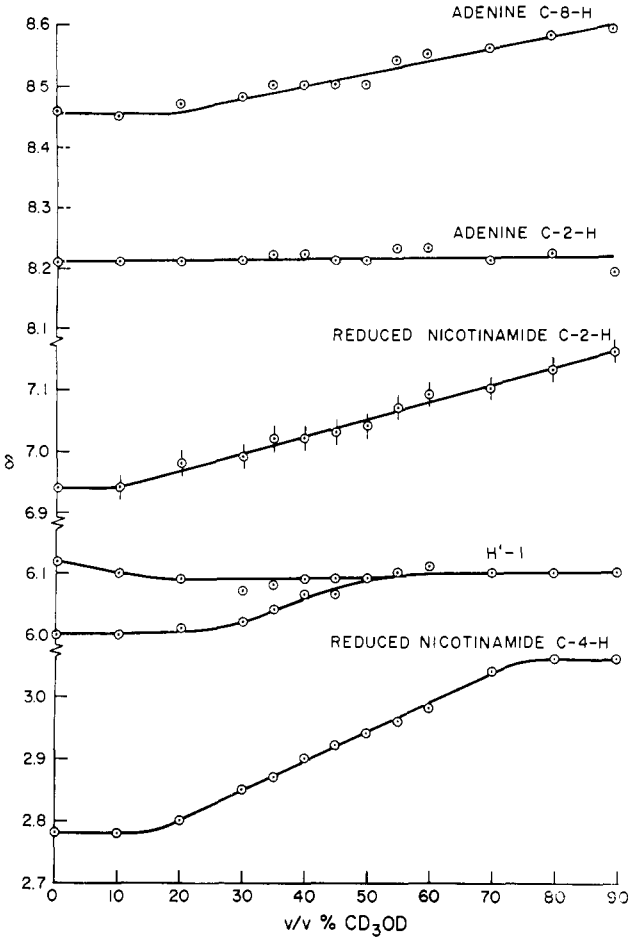

FIGURE 3: The effect of the concentration of methanol (CD_3OD) on the chemical shifts for the indicated protons of NADH. The precision of the individual points in this figure and Figure 4 is illustrated by the bars associated with the data for the chemical shifts of the proton attached to C-2 of the reduced nicotinamide ring.

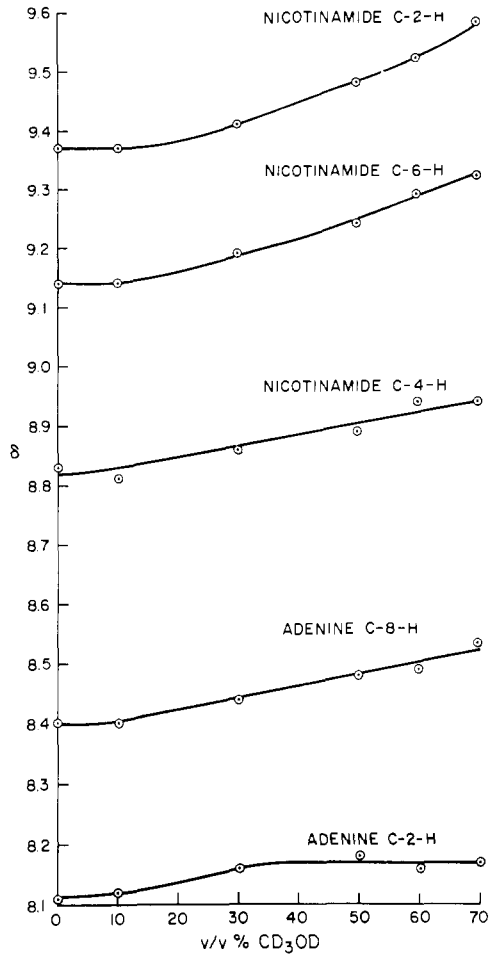

FIGURE 4: The effect of the concentration of methanol (CD_3OD) on the chemical shifts for the indicated protons of NAD.

Other workers (Moore and Underwood, 1969) have raised the question of whether the $pK_a = 3.88$ should be assigned to a phosphate or to the N-1 of adenine. The Jardetzkys' nuclear magnetic resonance studies of NAD indicated that protonation of the N-1 of adenine occurred between pH 3 and 4, and that this protonation resulted in unfolding. The results reported in this paper confirm that protonation of the adenine ring of both NAD and NADH occurs between pH 3 and 4. We feel that these results warrent assignment of the $pK_a = 3.88$ to the N-1 of adenine.

In Figure 3 appears the relationship between the chemical shifts for the various protons of NADH and the concentration of methanol. The C-2 and C-4 protons of the reduced nicotinamide ring show the most pronounced downfield shifts as the methanol concentration is increased. The direction of these shifts is the same as would be expected for progressive unfolding of NADH in increasing methanol concentrations (*i.e.*, as the polarity of the environment decreases). The C-2 proton of the adenine ring is independent of methanol concentration, but the C-8 proton shows a considerable downfield shift. Thus, while the results in Figure 2 indicate that downfield shifts can occur for both the C-2-H and the C-8-H when unfolding does not occur (*e.g.*, when the adenine ring of NADH is protonated), Figure 3 indicates that shifts need not occur for the C-2 proton when unfolding does occur. It is unknown whether or not the significant shift for the C-8 proton illustrated in Figure 3 is due to the unfolding. The C'-1 proton shifts show a small variation with methanol concentration. The higher field C'-1 resonance which is assigned to the ribose ring attached to adenine moves upfield when the methanol concentration is increased while the lower field C'-1-H resonance moves downfield.

It is interesting that the relationship between the downfield shifts due to unfolding and methanol concentration, and the relationship between fluorescence transfer and methanol concentration (Freed *et al.*, 1967) are quite different. The sharp dependence of the fluorescence transfer upon a narrow range of methanol concentration may reflect a requirement for proper (parallel?) positioning of the folded nucleotide rings for energy transfer for fluorescence emission. On the other hand, the nearly linear relationship between the downfield shifts and a wide range of methanol concentrations probably reflects a progressive unfolding of the nucleotide rings.

In Figure 4 appears the relationship between the chemical shifts for the various protons of NAD and the concentration of methanol. Spectra were not obtained above 70% methanol because of the low solubility of NAD in methanolic solutions.

TABLE I: Shifts in Parts per Million Relative to Internal DSS.

NAD^+	0.05 M	0.005 M	$\Delta\delta$	pH 7.3, 0.05 + 4 M Urea	$\Delta\delta$	pH 7.3, 0.05 + 8 M Urea	$\Delta\delta$
N-C-2	9.359	9.337	+0.022	9.389	−0.030	9.413	−0.054
N-C-6	9.165	9.154	+0.011	9.206	−0.041	9 232	−0.067
N-C-4	8.854	8.851	+0.003	8.902	−0.048	8.935	−0.081
N-C-5	8.212	8.194	+0.018	8.249	−0.037	8.263	−0.051
C'_1	6.118	6.106	+0.012	6.138	−0.020	6.179	−0.061
C'_1	6.038	6.043	−0.005	6.078	−0.046	6.100	−0.062
A-C-8	8.398	8.422	−0.024	8.408	−0.010	8.414	−0.016
A-C-2	8.114	8.182	−0.068	8.190	−0.076	8.223	−0.109
N-C-2	6.954	6.945	+0.009	6.993	−0.039	7.022	−0.068
N-C-4	2.776	2.776	0.000	2.855	−0.079	2.895	−0.119
C'_1	6.128	6.150	−0.022	6.144	−0.016	6.150	−0.022
C'_1	6.000	5.991	+0.009				
A-C-8	8.460	8.483	−0.032	8.444	+0.016	8.450	+0.010
A-C-2	8.209	8.249	−0.040	8.229	−0.020	8.285	−0.076

The data in Figure 4 illustrate that the C-2 and C-8 protons of the adenine ring and the C-2, C-4, and C-6 protons of the nicotinamide ring of NAD all experience downfield shifts when the methanol concentration is increased. These data are sufficient to show that NAD (like NADH) becomes progressively more unfolded as the methanol concentration increases. The larger shifts for the C-2 and the C-8 protons of the adenine of NAD (Figure 4) compared with protons in the same positions in NADH (Figure 3) are probably due to the fully aromatic pyridine ring in NAD; the greater diamagnetic anisotrophy of this ring would be expected to produce a greater effect on the adenine proton shifts than would occur in NADH. However, as in the case of NADH the adenine C-2 proton is affected less by unfolding than is the adenine C-8 proton. A similar effect is evident when the pH dependence of the chemical shifts of NAD and AMP are compared (Meyer *et al.*, 1962).

It can be seen from the data listed in Table I that urea also seems to produce unfolding of both NAD and NADH. The pyridine ring protons of both compounds show downfield shifts when the urea concentration is increased. Interestingly, in this case it is the adenine C-2 protons of both NAD and NADH that undergo a large downfield shift and the adenine C-8 protons that are nearly independent of urea concentrations. This situation is the opposite of that found in the case of unfolding caused by methanol. The chemical shift data for the adenine C-2 and C-8 protons in urea may be complicated by interactions of these protons with the strong hydrogen-bonding solvent. In spite of this the conclusion that unfolding occurs in aqueous urea solutions seems sound since the pyridine ring protons that cannot participate in hydrogen bonding shift substantially downfield.

The data in Figures 2 and 3 and Table I indicate two important points about the interpretation of the chemical shifts for the adenine C-2 and C-8 protons of NADH. First, unfolding can occur without significant change in the chemical shift of one or both of these protons. This may be related to the fact that the dihydropyridine ring has no ring current. Second, significant shifts can occur without unfolding due to perturbation of the ring electrons by protonations or other substitutions.

Finally, we would like to mention that we have found a definite concentration dependence of the chemical shifts of protons in both NAD and NADH in the concentration range of 0.05–0.005 M; this effect has not been previously detected. The data are illustrated in Table I. In light of this recent observation, it seems important to evaluate the effects of such intermolecular events on the chemical shifts of NAD and NADH in urea and methanol solutions. These investigations are in progress. It can be seen from Table I that only the adenine protons experience shifts due to changes of the concentration of NADH. In the case of NAD all of the pyridine ring protons except the C-4 proton show an upfield shift on dilution, but the adenine proton peaks shift downfield. Knowledge of the existence of this interaction is of practical importance since the resultant shifts are of the order of magnitude one would expect for the effect of enzyme binding on the chemical shifts if, for example, enzyme binding results in unfolding of the coenzymes.

The data reported in this paper indicate that both urea and methanol promote unfolding of NAD and NADH. Unfortunately we cannot draw concrete conclusions about the relative importance of the various factors which contribute to the stability of the folded conformation. The possibilities include hydrogen bonding, London dispersion forces, and hydrophobic interactions (Kauzman, 1959). Since linear hydrogen bonding between the nucleotide bases is not possible in the stacked conformation of the dinucleotides, very little contribution from this source is expected. Both London forces and hydrophobic interactions can be expected to make major contributions to the stability of the folded conformations. Both urea and methanol would be expected to weaken the hydrophobic interactions, and both could also affect London dispersion forces since they have different polarizabilities than water. Although the relative contributions of dispersion forces and hydrophobic interactions cannot be assessed from the present

data, we hope that our current studies of the temperature dependence of the unfolding will shed some light on this question.

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Contribution of Aromatic Residue Interactions to the Stability of Myoglobin. IV. Delineation of Binding Forces between Aromatic Compounds and Myoglobin*

John R. Cann

ABSTRACT: Aromatic compounds exert their enhancing effect on the rate of urea denaturation of myoglobin by complexing generally with two sites in the hemoprotein. The complexes are fundamentally of the electron donor–acceptor type with the aromatic compound acting as the donor, but hydrophobic interactions make a substantial contribution to their stability. Steric configuration of the aromatic compound and coulombic repulsion of donors with cationic side chains are also of importance. In fact, the relative rate enhancement of certain compounds is determined by a delicate balance of these forces. Whereas the urea denaturation of apomyoglobin is unaffected by a representative aromatic compound, reconstituted myoglobin shows the same sensitivity as myoglobin. Reversible complex formation has been demonstrated between hemin and β-naphthoate or 3-indolebutyrate and between hematoporphyrin and β-naphthoate. The above results are synthesized within the context of the three-dimensional structure of myoglobin with special reference to the heme moiety and its environs.

Finally, some interpretative comments are made on the band structure of spectra of model charge-transfer complexes.

As reported previously (Cann, 1965, 1967) aromatic compounds as diverse as benzene and chlorpromazine have an enormous and specific enhancing effect upon the rate of reaction of Zn^{2+} with myoglobin and the rate of urea denaturation of the protein. Kinetic and spectroscopic evidence has been advanced in support of our interpretation that these compounds exert their effect by forming electron donor–acceptor (charge-transfer) complexes with the heme moiety of myoglobin, the aromatic compounds serving as donors. Charge transfer is not the only force involved, however. Thus, there is an important hydrophobic contribution to the stability of the complex. Indeed, subsequent experiments suggested that in certain instances the balance of forces is such that hydrophobic interactions play a decisive role in determining the relative strength of complexing with the heme of two compounds having different electron-donating tendencies. A case in point is illustrated in Figure 1, which compares the effectiveness of benzene, naphthalene, and indole in enhancing the rate of urea denaturation of myoglobin. If charge transfer were the only force that need be considered, one would expect the effectiveness of these compounds to increase in the aforementioned order which is their order of increasing electron-donating tendency.[1] Ac-

* Contribution No. 368 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver, Colorado. *Received June 6, 1969.* Supported in part by Research Grant 5R01 AI01482-17 from the National Institutes of Health, U. S. Public Health Service.

[1] The theoretical ordering of these compounds as electron donors was verified experimentally by comparing the wavelengths of maximum absorption of their charge-transfer complexes with the acceptor molecule, chloranil, in carbon tetrachloride. The maximum wavelength of the charge-transfer band generally increases with decreasing molecular ionization potential of the donor, *i.e.*, with increasing tendency to donate electrons. The maximum wavelength and color of the complexes are: benzene, 347 mμ, yellow; naphthalene, bimodal with maxima at 384 and 478 mμ, orange; indole, 505 mμ, magenta. The benzene and naphthalene data are given in Table 7, p 30 of Briegleb (1961), the latter being confirmed in this investigation; indole, this investigation.