istry Department, and to Dr. Emil Kazes, Physics Department, for valuable discussion concerning the origin of the variation in the ΔTE values. We wish particularly to thank Professor R. S. Mulliken, University of Chicago, for helpful criticism.

Experimental

Materials.—The preparation, purification and properties of 1-methyl- and other alkylpyridinium iodides are described in detail elsewhere.[12]

1-Methyl-2-cyanopyridinium Iodide.—A solution of 24.61 g. (0.236 mole) of 2-cyanopyridine (Aldrich Chemical Co., Milwaukee) and 30 cc. (0.48 mole) of methyl iodide in 40 cc. of nitrogen-flushed dry methanol was allowed to stand at room temperature for twenty-one days. The salt crystallized out as orange prisms. Purification was accomplished by dissolving the rather pure first product in water (1 cc./g.), adding acetone (10 cc./g.), and cooling to 0°. Orange platelets were filtered off; yield 31.0 g. (61.8%), m.p. 175–176° (vac., dec.) (reported as yellow crystals, m.p. 183–184°).[21] Equiv. wt., calcd., 246.1; found, 245.6.

1-Methyl-2-carbethoxypyridinium Iodide.—Ethyl picolinate (Fluka A.G., Buchs, St. Gallen, Switzerland) was treated with methyl iodide in absolute ethanol, producing bright yellow needles from isopropyl alcohol, m.p. 108.3–109.3° (to red liq.). *Anal.* Calcd.: C, 36.88; H, 4.12. Found: C, 36.86; H, 4.28.

1-Methyl-3-carbomethoxypyridinium Iodide.—Methyl nicotinate (gift of Nepera Chemical Co., Inc., Yonkers, N.Y.) reacted with methyl iodide in methanol, giving light yellow crystals from methanol–ether, m.p. 132.5–133.5° (reported 129.5–130.2°),[22] equiv. wt., calcd., 279.2; found, 279.1.

Both 4-substituted pyridinium iodides listed in Table III have been described before.[2]

Tetraethylammonium triiodide, m.p. 139–141°, was recrystallized from ethanol (reported m.p. 142°[23]).

Acetone was dried by distillation from 4A Molecular Sieve (Linde Products) and water was distilled from potassium permanganate solution.

Spectra.—Potassium iodide in water and sodium iodide in acetone were measured with a Cary Recording Spectrophotometer, Model 14, using 0.0102 cm. cells (matched). The maxima were reproducible and offered no particular difficulties in their determination.[14]

All other spectra were obtained with a Cary, Model 11, Spectro grade methylene chloride (freshly opened!) and acetonitrile (Matheson, Coleman and Bell) served as solvents. Ethylene dichloride was carefully purified[19] and the chloroform had an alcohol content of *ca.* 0.90% unless otherwise indicated. Extraordinary care was exercised in the measurement of the data collected in Tables I and II and will be reported in detail in the accompanying publication.[12]

---

[21] R. I. Ellin, THIS JOURNAL, **80**, 6588 (1958). Repetition of the procedure used by Ellin (which is, in fact, the usual quaternization procedure) consistently gave 4% yield, not 36% as reported. In addition, some hydrogen cyanide is evolved during reflux and represents a potential hazard to the considerable number of people (perhaps 1 in 10) who are not able to detect this substance by its "characteristic odor."

[22] L. Bradlow and C. A. VanderWerf, *J. Org. Chem.*, **16**, 1143 (1951).

[23] S. K. Ray and D. Majumdar, *J. Indian Chem. Soc.*, **14**, 197 (1937).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN]

Pyridinium Complexes. II. The Nature of the Intermediate in the Dithionite Reduction of Diphosphopyridine Nucleotide DPN

BY EDWARD M. KOSOWER AND SONIA W. BAUER

RECEIVED JULY 30, 1959

The yellow intermediate formed in the dithionite reduction of DPN to DPNH is postulated to be a charge-transfer complex $DPN^+ \leftarrow SO_2^-$, on the basis of comparison of the spectra of intermediates derived from both 3- and 4-carbamidopyridinium ions and sodium dithionite.

One of the most important non-enzymatic reactions of the coenzyme DPN is the sodium dithionite reduction[1] to *enzymatically active* DPNH discovered by Warburg and his co-workers.[2,3] Other reducing agents, *e.g.*, electrolysis,[4] X-rays[5] and sodium borohydride,[6] attack the pyridinium ring to form substances of low or zero coenzymatic activity. It is now firmly established that enzymatically active DPNH[7] is a 1,4-dihydropyridine derivative.[8,9]

Von Euler and his group found that DPN and sodium dithionite gave yellow solutions under fairly basic conditions[10] and Yarmolinsky and Colowick produced an elegant demonstration that the yellow color was due to an intermediate in the formation of DPNH, with the composition $DPN \cdot SO_2^-$, formed according to equation 1[11]

$$DPN^+ + S_2O_4^= \longrightarrow DPN \cdot SO_2^- + SO_2 \quad (1)$$

Yarmolinsky and Colowick formulated the yellow intermediate as a 1,4-dihydropyridine derivative (I), but an analysis of its ultraviolet spectrum (Fig. 1) suggested that this might not be correct.[12] More recently, Wallenfels and Schüly[13] have advanced a 1,2-dihydro structure on spectroscopic evidence for a crystalline "sulfinate" from dithionite and 1-(2,6-dichlorobenzyl)-3,5-dicarbamidopy-

---

[1] Supported in part by Grant E-1608 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

[2] O. Warburg, W. Christian and A. Griese, *Biochem. Z.*, **282**, 157 (1935).

[3] E. M. Kosower, S. W. Bauer and D. Goldman, Abstracts IV International Congress for Biochemistry, Vienna, September 1–6, 1958, No. 1-35.

[4] B. Ke, *Arch. Biochem. Biophys.*, **60**, 505 (1956); R. F. Powning and C. C. Kratzing, *ibid.*, **66**, 249 (1957).

[5] A. J. Swallow, *Biochem. J.*, **54**, 253 (1953).

[6] M. B. Matthews, *J. Biol. Chem.*, **176**, 229 (1948); M. B. Matthews and E. E. Conn, THIS JOURNAL, **75**, 5428 (1953).

[7] DPN, diphosphopyridine nucleotide, DPNH, dihydrodiphosphopyridine nucleotide.

[8] M. Pullman, A. San Pietro and S. P. Colowick, *J. Biol. Chem.*, **206**, 129 (1954).

[9] R. F. Hutton and F. H. Westheimer, *Tetrahedron*, **3**, 73 (1959).

[10] H. v. Euler, E. Adler and H. Hellström, *Z. physiol. Chem.*, **241**, 239 (1936); E. Adler, H. Hellström and H. v. Euler, *ibid.*, **242**, 225 (1936).

[11] M. B. Yarmolinsky and S. P. Colowick, *Biochim. et Biophys. Acta*, **20**, 177 (1956).

[12] E. M. Kosower, "The Enzymes," Vol. II, Academic Press, Inc., New York, N. Y., 1960, Chapter 13.

[13] K. Wallenfels and H. Schüly, *Ann.*, **621**, 178 (1959); *Angew. Chem.*, **70**, 471 (1958).

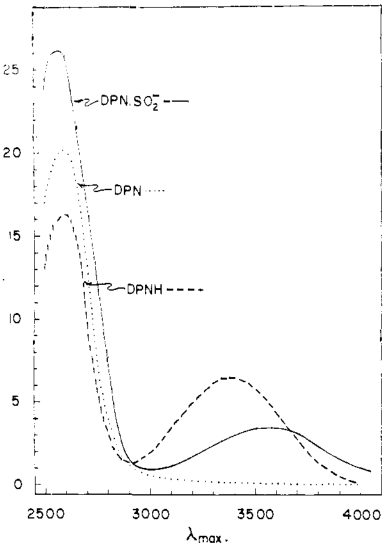
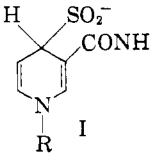
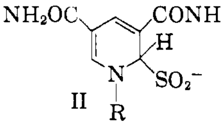


Fig. 1.—The absorption spectra of DPN, DPNH and the yellow intermediate, DPN·SO$_2^-$, in water. Curves were redrawn from those given in ref. 11.

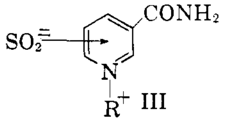
ridinium ion (II). However, the anion affinity[14] and electron affinity[15] of the pyridinium ring are extremely sensitive to substitution, and their formulation, although persuasive, cannot now be regarded as definitive for DPN. We wish to present evidence in favor of the view that the intermediate is a charge-transfer complex of sulfoxylate SO$_2^=$ and a pyridinium ion, expressed as III.



I



II



III

Results and Discussion

The investigation was based on the postulate that charge-transfer transitions for complexes with sulfoxylate as donor in the donor–acceptor complex[16] would show the same response to a change in substitution on the pyridinium ring as do iodide complexes. (A semi-quantitative expression for the transition energy differences, derived empirically from information in the literature, is given in the Appendix.) Since the position of pyridinium iodide charge-transfer absorption is extremely dependent upon the substitution in the pyridinium ion,[15,17] this criterion was chosen as the most sensitive test[12] of the spectroscopic data for the intermediate.

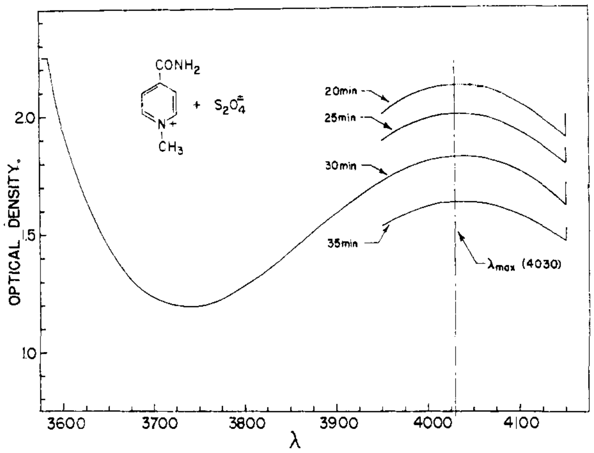


Fig. 2.—Behavior of 1-methyl-4-carbamidopyridinium sulfoxylate complex prepared from 1-methyl-4-carbamidopyridinium perchlorate (2.5 × 10^{-2} *M*) and sodium dithionite (2.5 × 10^{-2} *M*) in aqueous solution, *p*H 9.6.

The chemical nature of DPN governed the choice of the 3-carbamido group as one of the pyridinium ring substituents; the corresponding 4-carbamido group represented the minimum change in the nature of the substituent itself, but the maximum possibility for change in the position of the charge-transfer band.[18] The two pyridinium ions compared as acceptors in the present work are the 1-alkyl-3-carbamido (IV) and 1-alkyl-4-carbamidopyridinium (V) ions.

In order to establish the ΔE_T (*cf.* Appendix) expected for IV and V, spectra were measured on the iodides in a series of pyridine–water mixtures. The **Z**-values for the same solvents[17] also were measured with 1-ethyl-4-carbomethoxypyridinium iodide. Although the solvent range was limited for IV·Iodide, the positions of the charge-transfer bands (3700 to 3550 Å.) changed in a fashion parallel to **Z**, with a slope of *ca.* 0.92 on a E_T *versus* **Z** plot. The shift for V·Iodide, for which it was also possible to measure the charge-transfer maximum in dimethylformamide and dimethyl sulfoxide, was from 4075 to 3740 Å., corresponding to a slope of 0.96 in a E_T *versus* **Z** plot. This may be compared with the slope of 0.97 obtained for a plot of the transition energies of 1-ethyl-cyanopyridinium iodide charge-transfer band against a wide range of **Z**.[17] The data are given in Table IV, Experimental section. The average ΔE_T expected for 3- and 4-carbamidopyridinium ions on the basis of the iodide data is *ca.* 6.7 ± 0.3 kcal. mole.

Solutions of pyridinium ion, sodium dithionite and sodium hydroxide were prepared under nitrogen and their spectra measured. The intermediate formed rapidly in each case; however, the spectrum attributable to the intermediate in the case of the 4-carbamidopyridinium ions was somewhat evanescent (Fig. 2). The isonicotinamide analog of DPN prepared by Goldman and Bauer[19] gave rise to an intermediate which disappeared with such celerity that only a recording spectrophotometer made it possible to obtain the maximum. The results are summarized in Table I.

---

[14] K. Wallenfels and H. Diekmann, *Ann.*, **621**, 166 (1959).

[15] E. M. Kosower and J. A. Skorcz, "Proceedings of the Fourth European Molecular Spectroscopy Group Meeting," Bologna, Italy, September, 1959.

[16] R. S. Mulliken, THIS JOURNAL, **74**, 811 (1952); *J. Phys. Chem.*, **56**, 801 (1952).

[17] E. M. Kosower, THIS JOURNAL, **80**, 3253 (1958).

[18] E. M. Kosower and J. A. Skorcz, *ibid.*, **82**, 2195 (1960).

[19] D. S. Goodman and S. W. Bauer, to be published.

TABLE I

SODIUM DITHIONITE AND 1-ALKYL-3- AND 4-CARBAMIDOPYRIDINIUM IONS

Pyridinium ion	λ_{max} (Å.)	E_T (kcal./mole)	ΔE_T (kcal./mole)
1-Methyl-3-carbamido-	3730[a]	76.65	
1-Methyl-4-carbamido-	4030	70.94	5.7 ± 0.3
1-Ethyl-3-carbamido-	3720[a]	76.85	
1-Ethyl-4-carbamido-	4025	70.98	5.9 ± 0.3
DPN	3570[11]	80.08	
iso-DPN[b]	3870[a]	73.88	6.2 ± 0.4
		Value expected from iodides	6.7 ± 0.3[c]

[a] ±10 Å. [b] Isonicotinamide analog of DPN. [c] The slopes of the E_T *vs.* **Z** plots are such that ΔE_T would be smaller in aqueous solution.

TABLE II

PROPERTIES OF PYRIDINIUM SALTS

					Anal.			
					Calcd.		Found	
Pyridinium salt	X^-	M.p., °C.	λ_{max}, Å.	ϵ_{max}	C	H	C	H
1-Methyl-3-carbamido-	I^-	205.5–206.0	2648	4400		..[a,b]		
	ClO_4^-	145.3[c]	2649	4480	35.53	3.83	35.82	3.60
1-Methyl-4-carbamido-	I^-	255–256 (dec.)	2655	4840		..[d,e]		
	ClO_4^-	185.3[c]	2657	4720	35.53	3.83	35.76	3.82
1-Ethyl-3-carbamido-	I^-	202.5–202.8	2645	4760	34.55	3.99[a,f]	34.30	4.04
	ClO_4^-	139.5[c]	2648	4600	38.33	4.42	38.09	4.71
1-Ethyl-4-carbamido-	I^-	219.3–220.0	2655	4560	34.55	3.99[d]	34.75	3.99
	ClO_4^-	157.5[c]	2655	4700	38.33	4.42	38.62	4.56

[a] Light yellow crystals. [b] Reported 204°,[22,23] 202–204°,[24] 207–209°.[25] [c] Colorless crystals. [d] Bright yellow crystals. [e] Reported 255°,[26] 255–257°.[27] [f] Reported 188-189°,[24] 189°,[28] 198°[23,29] 198–199°,[30] 200°.[31]

Insofar as the postulate stated at the beginning of this section is valid, the results support formulation of the intermediate in dithionite reduction of pyridinium ring as a charge-transfer complex. The most probable structure for a complex, with one oxygen of the sulfoxylate group over the positively charged pyridinium nitrogen and the remainder of the ion over the pyridinium ring, places the second oxygen close to the 4-position of the pyridinium ring. (The isoelectronic (to SO_2^-) ClO_2^- ion has an OClO angle of 110°.)[20] It should be stated explicitly that this formulation of the intermediate III differs from the 2-adduct (II) of Wallenfels and Schüly in that it is a *nonlocalized* combination of the donor and acceptor.

It is known that the intermediate acquires a proton and loses sulfur dioxide in forming the dihydropyridine derivative.[11] The interpretation of the intermediate as a complex permits an attractive formulation of the pathway to the reduced product and is the only route which *predicts specific formation of a 1,4-dihydropyridine.* The key step is shown in Fig. 3. Other aspects of the possible role of complex formation in determining the position of nucleophilic attack on the pyridinium ring have been discussed previously.[21]

Experimental

Materials.—The 1-alkylpyridinium iodides were prepared by refluxing the appropriate alkyl iodide with nicotinamide or isonicotinamide in alcohol. The iodide salts were converted to the perchlorate salts by precipitating silver iodide with silver perchlorate in aqueous solution, followed by passing the solution through an ion-exchange resin (IR-45) column in the perchlorate form. Water was removed at reduced pressure and the solid recrystallized from 95% ethanol. The physical properties and analyses for new compounds are collected in Table II.

Solutions.—Water and other solvents used in this work were purged with high-purity nitrogen before use. In addition, it was found necessary to carry out most of the operations in a "low-oxygen" atmosphere, including dissolution of solids, dilution and transfers of solutions to cells. A stream of nitrogen from a funnel over the working area often was found to be adequate; in many cases, a tent made from polyethylene sheet and an aluminum frame and then filled with nitrogen provided more working space.

The purity of the sodium dithionite ("sodium hydrosulfite") was found to be *ca.* 85% by the method of Smith.[32]

Solutions of sodium dithionite in water are rather sensitive to oxygen. However, by excluding oxygen as much as possible, solutions could be prepared in 5 × 10^{-3} *N* sodium hydroxide which showed almost no change in absorption spectrum on standing for 14 hours at 25°, as measured by the curve near the maximum. We have found the maximum at 3160 Å., ϵ_{max} 6600–7000. In one case, a 1 × 10^{-4} *M* solution of sodium dithionite in 5 × 10^{-4} *N* sodium hydroxide had an initial ϵ_{max} of 8300 which decreased after one hour to 7000.[33] Although the intermediate derived from reaction of sodium dithionite with the 1-alkyl-3-carbamidopyridinium ion was moderately stable in 5 × 10^{-2} *N* base, primary attention was directed toward fixing the position of the maximum. For this purpose, a concentration of 5 × 10^{-4} *M* pyridinium ion was used most often. The instability and lower association constant of the intermediate from the 4-carbamido ion necessitated the use of higher con-

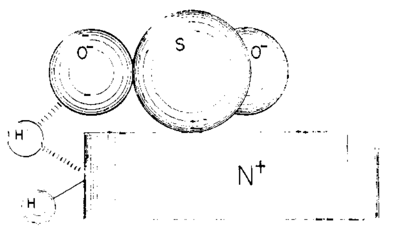


Fig. 3.—Transition state for the formation of a 1,4-dihydropyridine from a pyridinium sulfoxylate complex plus a proton.

---

[20] J. P. Mathieu, *Compt. rend.*, **234**, 2272 (1952).

[21] E. M. Kosower, THIS JOURNAL, **78**, 3497 (1956).

[22] P. Karrer, G. Schwarzenbach, F. Benz and U. Solmssen, *Helv. Chim. Acta*, **19**, 811 (1936).

[23] W. Ciusa, P. M. Strocchi and G. Adamo, *Gazz. chim. ital.*, **80**, 604 (1950).

[24] K. Tomita, *J. Pharm. Soc. Japan*, **71**, 220 (1951).

[25] E. M. Kosower and P. E. Klinedinst, Jr., THIS JOURNAL, **78**, 3493 (1956).

[26] P. Karrer, F. W. Kahnt, R. Epstein, W. Jaffe and T. Ishii, *Helv. Chim. Acta*, **21**, 223 (1938).

[27] C. A. Grob and E. Renk, *ibid.*, **37**, 1672 (1954).

[28] J. A. Gautier and J. Renault, *Compt. rend.*, **226**, 1736 (1948).

[29] P. Karrer and O. Warburg, *Biochem. Z.*, **285**, 297 (1936).

[30] M. Asano and K. Tomita, *J. Pharm. Soc. Japan*, **68**, 221 (1948).

[31] M. Rohrlich, *Arch. Pharm.*, **288**, 119 (1955).

[32] J. H. Smith, THIS JOURNAL, **43**, 1307 (1921).

[33] H. Hellström, *Z. physiol. Chem.*, **246**, 155 (1937), gives λ_{max} 313 mμ, ϵ_{max} 5900.

centrations, usually *ca.* 2.5 × 10^{-2} *M*, in solutions of lower *p*H (*ca.* 9.6), prepared by mixing 0.1 *N* sodium bicarbonate with an equal volume of 0.1 *N* sodium carbonate. Control experiments demonstrated that hydrolysis of the amide groups by hydroxide ion was relatively unimportant over the time periods required for the spectroscopic measurements. In 9.7 × 10^{-2} *N* base, the 1-methyl-4-carbamido- and 1-methyl-3-carbamidopyridinium ions hydrolyzed 24 and 14%, respectively, in 30 minutes, while in 2.4 × 10^{-2} *N* base the corresponding figures were 6 and 5%. Since the base concentration for the 3-ion never exceeded 5 × 10^{-2} *N*, hydrolysis can be disregarded in connection with the present work.

Spectra.—Cells (1.00 cm., 0.100 cm.) were filled under nitrogen with the solution and the measurements made as soon as possible thereafter with a Cary recording spectrophotometer, Model 11. The maxima obtained for the 1-methyl-4-carbamidopyridinium sulfoxylate complex in various solvents are listed in Table III.

TABLE III

1-METHYL-4-CARBAMIDOPYRIDINIUM SULFOXYLATE COMPLEX

Solvent[a]	λ_{max}
Water	4030
25% ethanol	4040
25% methanol	4080
25% acetonitrile	4080
50% ethanol	4120
50% methanol	4150
50% acetonitrile	4110
50% *t*-butyl alcohol	4140

[a] Volume per cent. mixture with water indicated.

"Iso-DPN."—A solution of the isonicotinamide analog of DPN[19] containing 7 mg. in 0.100 ml. of water was prepared; (C = 9.5 × 10^{-2} *M*). Aliquots of the iso-DPN solution (25 λ) were placed in a small centrifuge tube, and 0.400 ml. of 5 × 10^{-3} *M* sodium dithionite solution in 2.5 × 10^{-2} *N* sodium hydroxide was added rapidly. The mixture was transferred immediately to a microcell and the absorption spectrum measured in the ultraviolet. In three runs, maxima at 3880, 3865 and 3865 Å. were found with elapsed times (time of mixing to time at which maximum was passed) of *ca.* 90, 85 and 40 seconds, respectively. One solution was not measured until *ca.* 270 seconds had passed and no maximum could be found, although absorption due to intermediate obviously was present.

Reaction of Dithionite and 1-Methyl-4-carbamidopyridinium Ion.—Sodium dithionite and 1-methyl-4-carbamidopyridinium perchlorate were mixed in aqueous solution, allowed to stand a few hours and extracted with chloroform. The extract possessed an ultraviolet maximum around 2600 Å., and the residue after evaporation reduced silver nitrate. Only small amounts of chloroform soluble material were obtained and no characterizable compound was found in these preliminary experiments.

Iodide Charge-transfer Bands.—Solutions containing 96–100% pyridine (freshly distilled and dried) were prepared by pipetting the appropriate amounts of *previously thermostated* (25.2°) pyridine and water into a flask under nitrogen. The pyridinium iodides were 1-ethyl-3-carbamido- and 1-ethyl-4-carbamido- and 1-ethyl-4-carbomethoxy, the latter being used to establish the precise **Z** value[17] of the medium. The results are listed in Table IV.

TABLE IV

CHARGE-TRANSFER BANDS OF 1-ALKYLPYRIDINIUM IODIDES

Solvent[a]	λ_{max} (Å.) 1-Ethyl-3-carbamido	1-Ethyl-4-carbamido	1-Ethyl-4-carbomethoxy
100% pyridine[b]	3700	4075	4430
99.2%[c]	3653	4000	4335
98.4%[c]	3616	3935	4281
97.2%[c]	3550	3870	4197
DMF[d]	..[e]	3860	..[f]
96.0%[c]	..[e]	3815	4117
DMSO[g]	..[e]	3740	..[h]

[a] Volume per cent. pyridine. [b] 2 × 10^{-3} *M* pyridinium iodide. [c] 4 × 10^{-3} *M* pyridiniumi odide. [d] Dimethyl formamide. [e] Submerged. [f] Z = 68.5.[17] [g] Dimethyl sulfoxide. [h] Z = 71.1.[17]

Appendix

Reasonably linear relationships of charge-transfer band positions to the donor ionization potentials in donor–acceptor complexes have been reported for a number of cases.[34–36] Foster,[37] however, has shown that straight lines may be obtained for charge-transfer band positions when they are plotted against the positions for a "standard" complex (in his case, aromatic hydrocarbons and chloranil). Inspection of the plots made by Foster shows that, as a rough approximation, the variations in charge-transfer band positions are more or less parallel for a series of acceptors and different donors.[38]

For a pyridinium ion acceptor A_i, and a donor D_n, with the ionization potential I_n, equation 2 may be written for the transition energy

$$E_T\,(D_n \longrightarrow A_i) = mI_n + b_i \qquad (2)$$

A similar equation can be written for another pyridinium ion, A_j

$$E_T\,(D_n \longrightarrow A_j) = mI_n + b_j \qquad (3)$$

By subtracting equation 3 from equation 2, equation 4 can be obtained

$$E_T\,(D_n) = b_i - b_j \qquad (4)$$

Thus, for any donor, *the difference in transition energies for complexes formed with a pair of pyridinium ions should be a constant.*

---

(34) H. McConnell, J. S. Ham and J. R. Platt, *J. Chem. Phys.*, **21**, 66 (1953).

(35) S. H. Hastings, J. L. Franklin, J. C. Schiller and F. A. Matsen, THIS JOURNAL, **75**, 2900 (1953).

(36) R. E. Merrifield and W. D. Phillips, *ibid.*, **80**, 2778 (1958).

(37) R. Foster, *Nature*, **181**, 337 (1958).

(38) A similar conclusion has been reached by N. H. Smith, Ph.D. Thesis, University of Chicago, 1955.