The absence of any detectable absorption in these regions as well as in the vicinity of the $-CH_2-$ group resonance at -0.13 places an upper limit of 5 to 10% on the presence of form II.

As to the spectra at higher temperatures, we did not detect any reversible changes. There is, of course, the possibility that the large irreversible changes shown in Fig. 3 could have obscured a small reversible effect. Moreover, the temperature dependence of the infrared spectrum was observed[5] in the vapor phase where intermolecular reaction would be slower than for the liquid used in our experiments. So we cannot prove the absence of a small percentage of another form in equilibrium with V at temperatures above 120°. But this appears to us to be rather unlikely. For one thing, the arguments of Miller and Koch[5] supporting an equilibrium mixture imply comparable percentages of at least two forms at room temperature; and we have shown this definitely not to be the case. The changes with temperature reported[5] in the relative intensities of the infrared bands in the double bond region are comparable to those found at room temperature upon changing solvents.[4] Both observations can be ascribed to a dependency of the infrared transitions probabilities on molecular environment. A last point is that the new infrared bands found[5] at 180° could be spurious. New bands were also found at 100° upon heating the sample but disappeared or decreased in intensity upon further heating or upon heating to 180° and then cooling to 100° again. There is no assurance that the new bands found at 180° might not also have changed upon such treatment.

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[CONTRIBUTION FROM THE QUARTERMASTER RESEARCH & DEVELOPMENT CENTER]

Spectroscopic Studies on Dyes. I. The Association of Indigo Dyes in the Solid Phase[1]

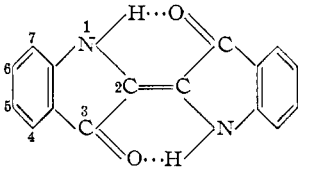
BY JULIUS WEINSTEIN AND GEORGE M. WYMAN

RECEIVED NOVEMBER 3, 1955

The visible spectra of indigo and seven of its alkyl- and halogen-substituted derivatives were determined in the solid phase as pellets in potassium bromide and also as aqueous suspensions. The first absorption band of indigo was found to exhibit a strong bathochromic shift when the solid spectrum was compared with that obtained in solution. The introduction of methyl or chlorine substituents in the 4- and 7-positions was found to decrease the magnitude of this shift. No such shift was observed in 5,5',7,7'-tetrabromoindigo. On the basis of these observations it was concluded that, in the solid state, indigo dyes are associated, probably by means of hydrogen-bonding. Bulky substituents in the 4,4'- or 7,7'-positions prevent the close approach of the –CO– and –NH– groups of neighboring molecules and thus interfere with the formation of molecular aggregates. A study of the infrared spectra of these dyes in the –CO– and –NH– stretching regions also supports this explanation. The absorption spectra of cellophane and gelatin films dyed with indigo indicate that under these conditions indigo is also present in the associated form.

Introduction

The problem of the structure and the configuration of indigo has intrigued organic chemists since Baeyer's classic work on the synthesis and constitution of this dye.[2] This has resulted in a large number of frequently conflicting reports in the literature on the subject.[3] There appears to be general agreement that indigo (I) is the *trans* isomer, preferentially stabilized by intramolecular hydrogen-bonding (which is only possible in the *trans* configuration), as had first been suggested by Scholl.[4] This was recently confirmed by spectrophotometric techniques when it was observed that, in contrast to thioindigo dyes,[5] indigo dyes in solutions in organic solvents do not undergo photochemical *trans-cis* isomerization.[6] In addition, the same authors also found evidence for hydrogen-bonding in indigo in the solid phase from a study of its infrared spectrum.



During a study of the effect of solvents on the spectrum of indigo, Sheppard and Newsome determined the visible spectrum of a solid film of this dye sublimed on glass.[7] They found that the absorption band occurs at considerably longer wave lengths in the solid phase than in solutions in organic solvents. This bathochromic shift is readily seen from the curves in Fig. 1. Since this unusually large shift in the position of the absorption band suggests the occurrence of structural changes, it was decided to undertake a study of the spectra of indigo and some of its derivatives in the solid phase in the hope of obtaining a better understanding of this phenomenon.[8]

Experimental

(a) **Dyes.**—4,4'-Dichloro-5,5'-dibromoindigo and 7,7'-dimethylindigo were research samples provided through

(1) Presented before the 127th Meeting of the American Chemical Society, Cincinnati, Ohio, April, 1955.

(2) A. v. Baeyer, *Ber.*, **16**, 2204 (1883).

(3) (a) T. Posner, *ibid.*, **59B**, 1799 (1926); (b) R. Pummerer and H. Fiesselmann, *Ann.*, **544**, 206 (1940); (c) G. Heller, *Ber.*, **77B**, 163 (1944); (d) J. v. Alphen, *Rec. Trav. Chim.*, **60**, 138 (1941).

(4) *Cf.* W. Madelung and O. Wilhelmi, *Ber.*, **57**, 237 (1924).

(5) (a) G. M. Wyman and W. R. Brode, THIS JOURNAL, **73**, 1487 (1951); (b) W. R. Brode and G. M. Wyman, *J. Research Natl. Bur. Standards*, **47**, 170 (1951).

(6) W. R. Brode, E. G. Pearson and G. M. Wyman, THIS JOURNAL, **76**, 1054 (1954).

(7) S. E. Sheppard and P. T. Newsome, *ibid.*, **64**, 2937 (1942).

(8) Shortly after the results of the present investigation were first reported it came to our attention that a study of the X-ray diffraction pattern of indigo disclosed that in the solid phase indigo exists in the *trans* configuration with association between neighboring dye molecules, probably by means of hydrogen bonding (*cf.* Helene v. Eller, *Compt. rend.*, **239**, 975 (1954)).

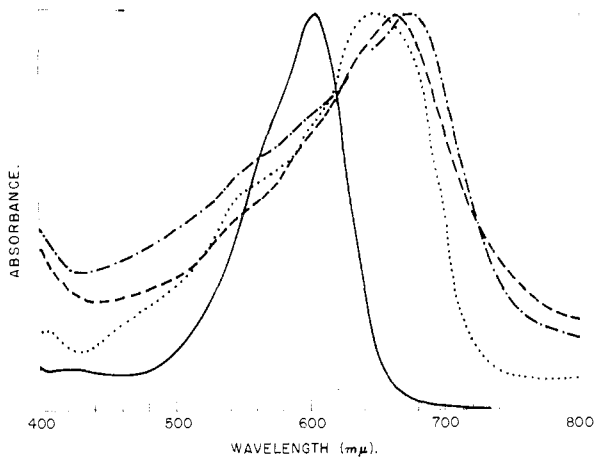


Fig. 1.—The visible spectrum of indigo (.....) as a sublimed film[7]; (———) in chloroform solution; (– – – –) as a pellet in KBr, and (–·–·–·) in aqueous suspension.

the courtesy of Dr. D. S. Davies of Imperial Chemical Industries, Ltd., Manchester, England; 5,5′- and 7,7′-difluoroindigos were research samples kindly provided by Prof. Arthur Roe of the University of North Carolina. N,N′-Diacetylindigo was prepared as described by Liebermann and Dickhuth.[9] The other dyes used in this work were commercial samples of known structure.

(b) **Preparation of Samples for Measurement.**—In the visible region the solid phase spectra were determined by two techniques: (1) as pressed pellets made from an intimate mixture of the dye with KBr[10]; and (2) as aqueous dispersions.[11] For measurements on solutions in this region and for all the infrared measurements standard techniques of sample preparation were utilized.

(c) **Measurement of the Absorption Spectra.**—The visible spectra were measured by means of a Cary Model 11 quartz spectrophotometer with the aid of the techniques described in references 5, 10 and 11. The infrared spectra were determined by means of a Beckman IR-3 spectrophotometer, using NaCl or LiF optics, as appropriate. For the measurement of KBr pellets a pellet-holder made of an opaque material was constructed which also acted as a diaphragm, since the pellets were not as wide as the measuring beam of the instrument. A pellet of pure KBr was used as the reference in these measurements.

Discussion of Results

The absorption bands found in the visible spectra of the indigo dyes that comprised this investigation are listed in Table I. The data on the solutions have been taken from reference 6, with the exception of 7,7′-difluoroindigo (which had previously been considered to be too insoluble for measurement) and 7,7′-dimethylindigo. Measurements were made on aqueous suspensions as well, although the potassium bromide technique usually gives clearer spectra, in order to eliminate the possibility of a reaction with potassium bromide or any effect of the pressure which is used in making the pellets.[12] It is evident from the data in Table I that the measurements obtained in the solid phase by the two techniques are in good agreement. In general, although the absorption curves obtained on the solid samples exhibit much broader maxima than those obtained on solutions, the absorption bands are readily located with an uncertainty of but a few millimicrons; this is shown in Figs. 1 and 2.

TABLE I

ABSORPTION BANDS OF INDIGO DYES IN THE VISIBLE REGION

Substituted indigo	Wavelength, mμ[a] In $CHCl_3$	In KBr	In aq. suspension
Indigo	604	660	668
5,5′-Difluoro-	618	686	681
7,7′-Difluoro-	619	683	680
7,7′-Dimethyl-	613	(600), 642	(607), 646
4,4′-Dichloro-	605	(590), 640	(600), 643
5,5′-Dibromo-	611	(612), 655	(617), 662
4,4′-Dichloro-5,5′-dibromo-	611	(610), 648	(612), 650
5,5′,7,7′-Tetrabromo-	615	614	607
N,N′-diacetyl-	559	558	559

[a] Major inflection points are indicated in parentheses

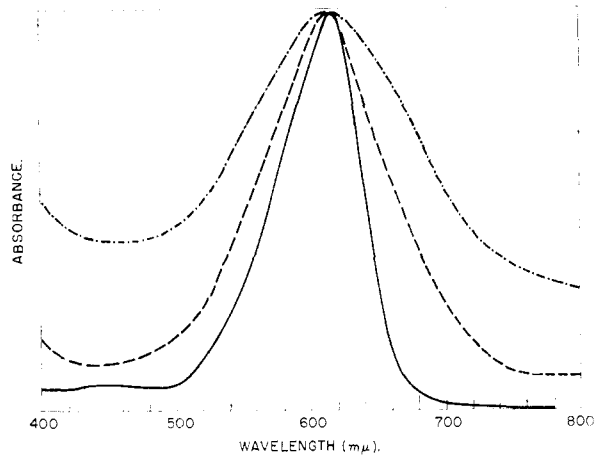
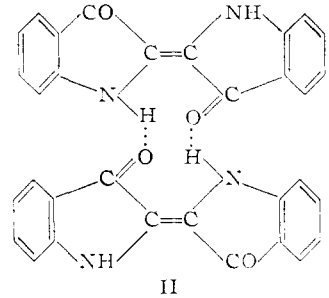


Fig. 2.—The visible spectrum of 5,5′,7,7′-tetrabromoindigo (———) in chloroform; (– – – –) as a KBr pellet, and (–·–·–·) in aqueous suspension.

It is apparent from Table I that the strong bathochromic shift first noted for indigo in the solid phase[7] is common to indigo dyes with the exception of those which contain bulky substituents in the 4- and 7-positions. A shift of this magnitude is usually indicative of a considerable extension of the resonating system, suggesting the formation of dimers or higher polymers in the solid phase, held together by intermolecular hydrogen-bonds, as shown in structure (II). In accordance with this picture, bulky substituents in the 4- and 7-positions



II

(9) C. Liebermann and F. Dickhuth, *Ber.*, **24**, 4131 (1891).

(10) G. M. Wyman, *J. Opt. Soc. Am.*, **45**, 965 (1955).

(11) K. Shibata, A. A. Benson and M. Calvin, *Biochim. et Biophys. Acta.* **15**, 461 (1954).

(12) Such a reaction has recently been reported to occur with thiourea. (*Cf.* J. E. Stewart, Abstracts of the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Pittsburgh, Pa., 1955, p. 34.)

would be expected to interfere with the close approach of the —CO— and —NH— groups of neighboring molecules and thus prevent association; the larger the substituent group the more effectively it will prevent association. The data in Table I indicate that, while fluorine atoms are not large enough to cause steric hindrance to association, the larger chlorine atoms and methyl groups give rise to some hindrance and the still larger bromine atoms in the 7- and 7′-positions are fully effective in preventing the formation of intermolecular hydrogen bonds. These conclusions were further substantiated when it was found that N,N′-diacetylindigo, a compound that does not contain hydrogen atoms that could be available for hydrogen-bonding, failed to exhibit any such shift. The observed increased solubility of 5,5′,7,7′-tetrabromoindigo, N,N′-diacetylindigo and N,N′-dimethylindigo,[13] as compared with indigo, is also consistent with this picture.

A study of the infrared spectra of these dyes provided further confirmation of these conclusions, as shown in Table II. In the carbonyl region (near 6 μ) each of the ring-substituted indigo dyes

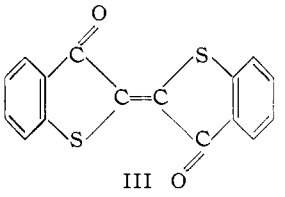
TABLE II

ABSORPTION BANDS OF INDIGO DYES IN THE INFRARED REGION

Substituted indigo	Wave length, μ –CO– band[a]	–NH– band[b]
Indigo	6.13, 6.19	3.06
5,5′-difluoro-	6.16	3.03
7,7′-difluoro-	6.15	3.06
7,7′-dimethyl-	6.12	2.93
4,4′-dichloro-	6.12	2.98
5,5′-dibromo-	6.12	3.03
4,4′-dichloro-5,5′-dibromo-	6.12	2.98
5,5′,7,7′-Tetrabromo-	6.04	2.97
Thioindigo	6.04	

[a] As potassium bromide pellets, using sodium chloride optics. [b] As Nujol mulls, using lithium fluoride optics.

exhibits a single intense absorption band, in contrast with the parent compound which shows two bands in this region.[14] It is apparent from these data that in all these indigo dyes with the exception of 5,5′,7,7′-tetrabromoindigo the carbonyl frequency occurs at longer wave lengths than in thioindigo (III). Since the environment of the



III

carbonyl group in the thioindigo molecule is much like that in indigo, the shift to longer wave lengths in the latter must be attributed to hydrogen bonding. The occurrence of the carbonyl band in 5,5′,7,7′-tetrabromoindigo at the same wave length as in thioindigo suggests that in this molecule the hydrogen bonding is weaker than in the other indigo dyes. (Some of this shift to shorter wave lengths may also be attributable to the effect of the accumulation of negative halogen atoms in the aromatic rings; *e. g.*, a hexachloro derivative of thioindigo was found to have its carbonyl band at 6.02 μ.) It is unfortunate that the low solubility of indigo dyes does not permit comparisons between the spectra of the solids and of their solutions, since then it would be an easy matter to differentiate between intra- and intermolecular hydrogen bonding from a study of the carbonyl region in the infrared spectra.

In order to help in ascertaining the configuration of these dyes in the solid phase, the infrared spectra of solutions of some of the more soluble of the structurally related thioindigo dyes were studied with the hope that the carbonyl absorption bands of the *cis* and *trans* isomers would be found at different wave lengths. This was considered probable, since it had previously been shown that the carbonyl bands occur at different frequencies in the *cis* and *trans* isomers of 1,2-dibenzoylethylene.[15] Accordingly, the chloroform solutions of two thioindigo dyes were enriched with respect to the *cis*-isomers by irradiating them with yellow light, as described in reference 5. The infrared spectra of these solutions in the carbonyl region are shown in Fig. 3. It is apparent from these curves that *trans* to *cis* isomerization does cause a decrease in the intensity of the carbonyl bands of the *trans* form (near 6.06 μ) and gives rise to new absorption bands at shorter wave lengths (near 5.90 μ). The new band is bound to be the carbonyl absorption band of the *cis* isomer. By analogy the carbonyl band of *cis*-indigo and its derivatives would also be expected to occur at wave lengths shorter than 6 μ. Since in every one of the indigo dyes studied in this work the carbonyl band occurs at a wave length longer than 6 μ, it is then possible to conclude that in the solid phase they exist in the *trans*-configuration.

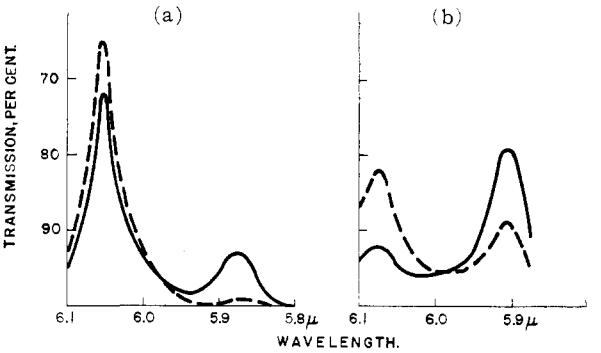


Fig. 3.—Infrared spectra obtained for (a) thioindigo and (b) 6,6′-diethoxythioindigo in chloroform solution after irradiation with blue (– – – –) and yellow (———) light, respectively.

A study of the —NH— stretching frequencies (near 3 μ) of these dyes reveals further evidence in favor of the above conclusions. The spectra of indigo, the two difluoroindigos and 5,5′-dibromoindigo (*i. e.*, the dyes that showed the largest solid-

(13) L. Ettinger and P. Friedlaender, *Ber.*, **45**, 2074 (1912).

(14) With the high resolving power of the IR-3 instrument it was possible to resolve the band at 6.17 μ reported in reference 6.

(15) L. P. Kuhn, R. E. Lutz and C. R. Bauer, THIS JOURNAL, **72**, 5058 (1950).

to-solution shift in the visible spectra) exhibit a broad band in this region at a wave length slightly longer than 3 μ (*cf.* Table II). The remaining dyes, containing bulky substituents in the 4,4′- or 7,7′-positions show a sharp band at a wave length somewhat shorter than 3 μ. This suggests that in those indigo dyes where the —NH— groups partake in intermolecular hydrogen-bonding, the —NH— stretching frequency occurs at slightly longer wave lengths than that observed in the dyes in which the hydrogen-bonding is mainly intramolecular. A similar difference in the —NH— stretching frequencies has also been observed by Blout in a study of the N,N′-dialkylamides of maleic and fumaric acids where, because of their *cis* configuration, only the former are capable of forming intramolecular hydrogen bonds.[16]

In view of the importance of indigo and some of its derivatives as dyestuffs, it is of interest to note that cellophane and gelatin films dyed with indigo also exhibited absorption maxima at 662 mμ, indicative of the presence of the associated form. Since cellophane and gelatin are structurally similar to cotton and wool, respectively, this strongly suggests that it is the polymeric form that is present in fabrics dyed with indigo. This further deepens the mystery surrounding the nature of the forces that hold dyes of this type to fibers, since it is difficult to envisage a mechanism by which indigo molecules, involved as they are in both intra- and intermolecular hydrogen-bonding, are bound to fibers.

Acknowledgment.—The authors wish to record their appreciation to Dr. E. R. Blout who suggested the study of the —NH— stretching frequencies for evidence of intermolecular hydrogen-bonding and to Mrs. D. A. Rogers for carrying out the study of carbonyl frequencies of the two thioindigo dyes included in this paper.

(16) E. R. Blout, private communication.

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, ROHM AND HAAS COMPANY]

The Kinetics of the Decolorization of Anthocyanins by Fungal "Anthocyanase"[1]

BY H. T. HUANG[2]

RECEIVED OCTOBER 3, 1955

Measurements of the decolorization of chrysanthemin at *p*H 3.95 and 30° by a fungal anthocyanase were analyzed kinetically. It was shown that the absorbancy of a decolorizing solution could be adequately described in terms of a_0, the initial absorbancy, t, the incubation time, k', an apparent first-order rate constant for the enzymatic hydrolysis of the glucoside, and k_1 and k_2, two apparent first-order rate constants involved in the reversible transformation of the liberated aglucone into a colorless derivative. k' is a function of enzyme and substrate concentrations. Values of k', k_1 and k_2 were estimated by independent methods and found to agree satisfactorily with those calculated from decolorization data.

Several crude enzyme preparations derived from *Aspergilli* have been found to exert a decolorizing effect on anthocyanins in aqueous solution, within a *p*H range of 3.0 to 4.5.[3] The over-all process was shown to involve firstly, an enzymatic hydrolysis of the anthocyanin to anthocyanidin and sugar, and secondly, a spontaneous transformation of the aglucone pigment into colorless forms. Information was inadequate to characterize clearly the enzyme responsible for the hydrolytic reaction. It was tentatively referred to as "anthocyanase." Detailed kinetic data on the decolorization of chrysanthemin by anthocyanase CN 558 are now available. It will be shown that the kinetics of decolorization are consistent with the above reaction scheme.

Experimental

Chrysanthemin chloride was a crystalline monohydrate isolated from blackberry as described previously.[3] It was shown to be indistinguishable from a synthetic sample of cyanidin-3-β-monoglucoside.[4] Anthocyanase CN 558 was the same preparation as that used in the preceding work.[3] Cyanidin chloride was prepared by hydrolysis of the glucoside in 20% hydrochloric acid.[3]

Decolorization experiments were carried out at 30° in 0.045 *M* sodium lactate buffer at *p*H 3.95. One ml. of enzyme solution was mixed with 5 ml. of substrate solution in buffer in a 12.5 × 125 mm. optically calibrated test-tube. The tube was stoppered and the absorbancy of the reacting solution at 510 mμ was read in a Coleman Junior Spectrophotometer against a lactate–enzyme blank. It was then incubated in a constant temperature bath maintained at 30 ± 0.05°. At selected intervals the tube was removed and a reading of the absorbancy at 510 mμ was again taken. Independent measurements had indicated that within the range employed, concentration of chrysanthemin was directly proportional to absorbancy at 510 mμ. When incubation under N_2 was desired, the tube was flushed well with N_2 during and after the addition of enzyme, and then tightly stoppered.

Reducing sugar was determined colorimetrically by a modification of Somogyi's micro method,[5] using the chromogenic reagent of Nelson.[6] The reducing capacity of chrysanthemin, cyanidin and glucose was found to be equivalent on a molar basis. In direct measurements of the extent of enzymatic hydrolysis, 1-ml. aliquot was mixed well with 1 ml. of Somogyi's reagent, and the mixture kept at 0° until a series of samples was ready for heating and subsequent treatment. The *p*H of the mixture was high enough (>9.7) to arrest all enzyme activity and the over-all procedure produced no chemical hydrolysis of the unchanged glucoside. The extent of hydrolysis was, therefore, proportional to the increase in reducing capacity found in the reacting solution.

Direct observations on the spontaneous decolorization of cyanidin were made by mixing concentrated solutions of cyanidin chloride at *p*H <1 with buffer at *p*H 3.95 at 30° and following the absorbancy of the solution at 510 mμ at regular intervals.

Results and Discussion

Results of most experiments, particularly when the level of enzyme employed was high, suggest that

(1) Presented in part before the Division of Biological Chemistry at the 126th Meeting of the American Chemical Society in New York, N. Y., September 12–17, 1954.

(2) Biochemical Research Laboratories, Chas. Pfizer and Co., Inc., Brooklyn, N. Y.

(3) H. T. Huang, *J. Agric. Food Chem.*, **3**, 141 (1955).

(4) H. T. Huang, *Nature*, **177**, 39 (1956).

(5) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).

(6) N. Nelson, *ibid.*, **153**, 375 (1944).