strength. Human γ_2-globulin shows a large amount of reversible spreading and the standard deviation for the mobility distribution has been found to vary with ionic strength in the direction expected from electrolytic solution theory.

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Photochemistry of Tryptophan, *p*-Dimethylaminobenzaldehyde and Reaction Products in Sulfuric Acid Solution[1]

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During a previously reported study[2] of the color-forming reactions between tryptophan, *p*-dimethylaminobenzaldehyde (DAB) and sodium nitrite in sulfuric acid solution, it became necessary to study the effects of light on the reactants and reactions involved. This paper describes a method for studying the effects of illumination (the term "illumination" refers to exposure to light by a procedure described below), the effect of light on the stability of tryptophan, the effects of light on reactions I and II,[3] and a photochemical "after effect" produced by illumination of DAB in acid solution. The reactions were carried out in 19 *N* sulfuric acid because this concentration was found suitable for the determination of tryptophan in proteins by a procedure to be described in later papers.

Photochemical development of color from the colorless condensation product of tryptophan and DAB was first reported by Boyd[4] who attributed this effect to the ultraviolet rays of sunlight.[5]

The effect of illumination on the stability of tryptophan in 19 *N* acid at 25° is shown in Table I. Light accelerates the decomposition of tryptophan as losses of 3, 11 and 34% occurred on illumination for one-half, one and three hours, respectively, compared with a loss of only 8% on standing in the dark for forty-eight hours.

TABLE I

EFFECT OF LIGHT ON THE STABILITY OF FREE TRYPTOPHAN IN 19 *N* SULFURIC ACID AT 25°[a]

Time illuminated or dark, hours	Loss of tryptophan, % Illuminated	Dark[b]
0	0	..
0.1	0	0
.25	0	..
.50	3	0
1	11	0
2	..	0
3	34	..
4	..	3
24	..	6
48	..	8

[a] Procedure: nine ml. of 21.4 *N* acid at 25° was placed in tube A, Fig. 2, and 100 γ of tryptophan in 1.0 ml. of water was added. The solution was mixed and tube A was placed at once in holder B through which water at 25 ± 0.1° was circulating. Illumination was started twenty seconds after adding the tryptophan to the acid solution. After the desired interval of illumination the solution was poured onto 30 mg. of DAB in a 25-ml. glass-stoppered Erlenmeyer flask. Tryptophan was then determined by procedure C, Paper I.[2] [b] Results taken from Table VII, Paper I[2].

The effects of illumination during reaction I under conditions such that reactions I and II were proceeding simultaneously are shown in Table II. Illumination for the first ten seconds of reaction I caused no increase in pre-nitrite color[6] nor any loss of tryptophan. But illumination for thirty seconds caused an increase in pre-nitrite color and destruction of 6% of the tryptophan. The pre-nitrite color increased with time of illumination until after 180 minutes it amounted to 76% of the total. The loss of tryptophan, caused by illumination, increased to 25% of the total during the first five minutes of reaction I and then remained constant. Reaction I is 87% completed in five minutes (Table VI).[2] Therefore the destructive effects of illumination occur chiefly during the condensation of tryptophan and DAB.

Rate of reaction II caused photochemically is rapid at first and then becomes quite slow as 70, 88 and 93% of the color was developed by five, twenty, and 120 minutes illumination, respectively.[7] After five minutes of illumination only 94% of the potentially available color could be obtained by subsequent oxidation with sodium nitrite. This destructive effect, however, was not progres-

(1) Paper II in a series entitled, "Chemical Determination of Tryptophan." Presented at the 111th meeting of the American Chemical Society held at Atlantic City, New Jersey, April, 1947. Not subject to copyright.

(2) Spies and Chambers, *Anal. Chem.*, **20**, 30 (1948).

(3) The condensation of tryptophan and DAB to form the leuco base is called reaction I and the oxidative development of the blue color, either photochemically or with sodium nitrite, is designated reaction II.

(4) Boyd, *Biochem. J.*, **23**, 78 (1929).

(5) Ruemele, *Z. Untersuch. Lebensm.*, **79**, 453 (1940), observed that light effected the formaldehyde–tryptophan colorimetric reaction but no detailed study was made.

(6) The term pre-nitrite color will refer to that color which develops in a test solution either spontaneously or as a result of exposure to light. The term post-nitrite color refers to that color which develops in a test solution after addition of sodium nitrite.

(7) Procedure: To 1.607 mg. of tryptophan and 482 mg. of DAB in a glass-stoppered Erlenmeyer flask was added 160.7 ml. of 19 *N* acid. The solution was kept in the dark at 25° for twenty-two hours. After illumination of 10 ml. aliquots of this solution pre-nitrite transmittancies were read and expressed as per cent. of the maximum color obtainable under ideal conditions of the test (procedure E, Paper I[2]).

TABLE II

EFFECT OF ILLUMINATION ON REACTION I[a]

Time illuminated	% Total color formed[b] Pre-nitrite	Post-nitrite	Loss
0	13	100	0
1 sec.	15	100	0
10 sec.	13	100	0
30 sec.	19	94	6
1 min.	29	89	11
5 min.	65	75	25
15 min.	68	72	28
30 min.	70	72	28
1 hr.	72	75	25
3 hr.	76	76	24

[a] Procedure: Eight ml. of 23.8 N acid and 1.0 ml. of 2 N acid containing 30 mg. of DAB were mixed and cooled to 25°. To this solution was added 100 γ of tryptophan in 1.0 ml. of water. Immediately after mixing, the solution was placed in tube A, Fig. 2, which was then placed in B, Fig. 2, through which water at 25 ± 0.1° was circulating. Illumination was started twenty seconds after adding the tryptophan. After the desired interval of illumination the solution was poured into a 25-ml. glass-stoppered Erlenmeyer flask which was stored in the dark at 25° for twenty to twenty-four hours. Pre-nitrite transmittancies were then read. Color was then developed by adding 0.1 ml. of 0.04% sodium nitrite solution. Post-nitrite transmittancies were read after the solutions had stood for thirty minutes in the dark. [b] Transmittancies were converted to weight of tryptophan then expressed as the per cent. of the maximum color obtainable with an equal quantity of tryptophan under ideal conditions of the test.[2]

sive because whether the illumination was for five or 120 minutes the same amount of color could subsequently be obtained with sodium nitrite.

Illumination of test solutions, in which the color had already been fully developed with sodium nitrite, for ninety minutes caused no loss in color intensity thus showing the stability of the blue colored substance to light.

The color formed photochemically has an absorption curve similar to that formed by sodium nitrite as shown in Fig. 1. The wave length of maximum density was 590 to 600 mu in both curves B and G and a characteristic absorption peak occurred at 425 mu.

Illumination of DAB in 21.4 N acid before the addition of tryptophan to the test solution produced considerable pre-nitrite color as "after effect."[8] The effect of time of illumination on the intensity of the pre-nitrite and post-nitrite colors is shown in Table III. The pre-nitrite colors of all non-illuminated control solutions of DAB represented from 2.5 to 4.5% of the total color. But when DAB was illuminated for five seconds, one, five, fifteen and sixty minutes, prior to addition of the tryptophan, the pre-nitrite color represented 5.1, 20, 45, 55 and 58% of the total color, respectively. Losses of tryptophan from 1.5 to 5.4%

(8) The photochemical "after effect" is discussed in some detail by Kistiakowsky, "Photochemical Processes," The Chemical Catalog Co., Inc., New York, N. Y., 1928, and Dhar, "The Chemical Action of Light," Blackie and Son, Limited, London and Glasgow, 1931. In general, this term refers to those photochemical reactions which occur after cessation of illumination.

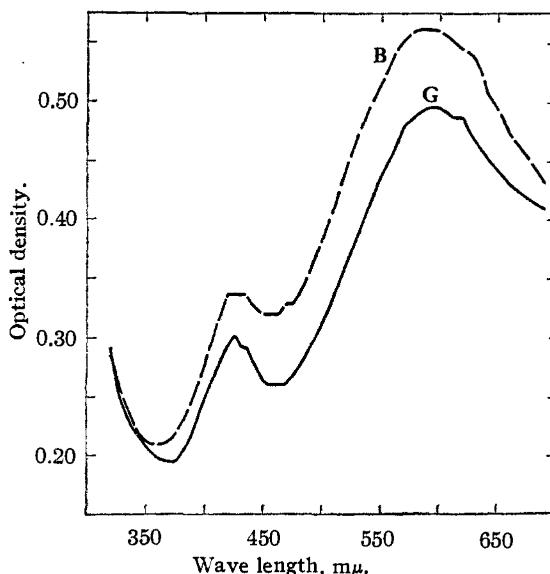

Fig. 1.—Absorption curves of tryptophan-DAB color developed with sodium nitrite (Curve B, taken from Fig. 1, Paper I) and light (Curve G, illuminated for 20 minutes). 100 γ of tryptophan was used for each test. Transmittancies were determined with a Beckman spectrophotometer with 10-mm. cuvettes.

TABLE III

EFFECT OF TIME OF ILLUMINATION ON THE PHOTOCHEMICAL AFTER EFFECT OF DAB IN 21.4 N ACID AT 25°[a]

Time illuminated	% Total color formed[b] Pre-nitrite Illuminated	Pre-nitrite Control	Post-nitrite Illuminated	Post-nitrite Control	Loss by illuminated
5 sec.	5.1	4.5	98.5	100	1.5
1 min.	20	3.2	96.8	100	3.2
5 min.	45	3.2	96.4	100	3.6
15 min.	55	2.5	94.6	100	5.4
60 min.	58	3.2	94.6	100	5.4

[a] Procedure for all tests was standardized so that the only variable was the interval of illumination. For each illumination test a control test was run in the same way except that the test was kept in the dark for a period of time equal to the illumination interval of the corresponding test. Following is an example of the test illuminated for five minutes and the control. Thirty mg. of DAB, in Tube A, was dissolved in 9.0 ml. of 21.4 N acid, and Tube A was placed in B through which water at 25 ± 0.1° was circulating. Two minutes after adding the acid the tube was illuminated for five minutes. Tube A was left in B for one minute after stopping the illumination and then 100 γ of tryptophan in 1.0 ml. of water was added to the test. The solution was mixed and poured into a 25-ml. glass-stoppered Erlenmeyer flask. The solution was cooled in the water-bath at 25° for two minutes and then placed in a dark chamber at 25° for four hours. Pre-nitrite transmittancy was then read using a control containing 30 mg. of DAB in 10 ml. of 19 N acid. To the solution was added 0.1 ml. of 0.04% sodium nitrite. Post-nitrite transmittancy was read after the test had stood thirty minutes in the dark. The control test was similar except that the solution was allowed to stand in Tube A in B in the dark for eight minutes before adding the tryptophan solution. [b] Transmittancy readings were converted to micrograms of tryptophan and then expressed as the per cent. of the maximum color obtainable with an equal quantity of tryptophan under ideal conditions of the test.[2]

resulted from using illuminated DAB solutions. These losses are attributed to the oxidative action of illuminated DAB on tryptophan before completion of reaction I a time when tryptophan is very sensitive to oxidative destruction as previously shown.

The rate of pre-nitrite development of color which resulted when tryptophan was added to previously illuminated solutions of DAB in 21.4 N acid is shown in Table IV. The pre-nitrite color of control tests ranged from 2.4 to 13% in four to seventy-one hours while the pre-nitrite color in tests in which DAB had been illuminated increased from 19% in fifteen minutes to a maximum of 71% in forty-seven hours. Eight to 10% of the tryptophan originally present was destroyed by the illuminated DAB solutions.

TABLE IV

RATE OF COLOR DEVELOPMENT RESULTING FROM PHOTOCHEMICAL AFTER EFFECT CAUSED BY ILLUMINATION OF DAB IN 21.4 N ACID AT 25°[a]

Time after addition of tryptophan to reading of pre-nitrite transmittancy, hours	% Total color formed[b]				Loss by illumination, %
	Pre-nitrite		Post-nitrite		
	Illuminated	Control	Illuminated	Control	
0.25	18				
1	34				
2	44				
4	55	2.4			
24	71	6.6	92	100	8
47	71	11			
71	69	13	86	96	10

[a] Procedure for all tests was standardized so that the only variable was the interval between addition of the tryptophan to the illuminated solution of DAB and reading the pre-nitrite transmittancy. For illumination experiments in which the time was four, twenty-four, forty-seven and seventy-one hours control tests were run in the same way except that illumination of DAB was omitted. Following is an example of the twenty-four-hour test: 30 mg. of DAB was placed in Tube A, Fig. 2, and dissolved in 9.0 ml. of 21.4 N acid. Tube A was then placed in B at 25° for two minutes. The solution was then illuminated for fifteen minutes and after standing one minute more 100 γ of tryptophan in 1.0 ml. of water was added. The solution was mixed and poured into a 25-ml. glass-stoppered Erlenmeyer flask which was then cooled in the water-bath at 25° for two minutes. The flask was placed in a dark chamber at 25° for twenty-four hours. Pre-nitrite transmittancy was then read. A blank solution containing 10 ml. of 19 N acid was used for all tests. To the solution was then added 0.1 ml. of 0.04% sodium nitrite solution and after standing in the dark for thirty minutes post-nitrite transmittancy was determined. [b] Transmittancy readings were converted to micrograms of tryptophan and then expressed as the per cent. of the maximum color obtainable with an equal quantity of tryptophan under ideal conditions of the test.[2]

In another series of experiments it was shown that pre-nitrite color of the same intensity was obtained whether the tryptophan was added five seconds or twenty hours after stopping the illumination of DAB. Therefore, once a solution of DAB has been exposed to light it appears to retain its oxidative capacity indefinitely.

Discussion

The mechanism of the photochemical development of color from the tryptophan–DAB condensation product probably involves oxidation by dissolved oxygen through the medium of DAB in the role of oxygen acceptor. The primary process is probably the formation of the peracid of DAB which then oxidizes the tryptophan–DAB complex to form the blue-colored compound. Cole[9] noted that color slowly developed without addition of an oxidizing agent when an acid solution of benzaldehyde and tryptophan stood a few days. Cole did not mention the possible effect of light in this reaction but believed that oxidation resulted from benzoyl peroxide formed by atmospheric oxygen. Bäckström,[10] who studied the photochemical oxidation of benzaldehyde and heptanal, postulated a chain mechanism for the photochemical formation of the peracids because the quantum yield was very large. According to Bäckström the quantum yield of benzoic acid was nearly constant in the region of 2536 to 3660 Å. Boyd[4] believed the photochemical effect observed by him was caused by ultraviolet light. The present experiments indicate that photochemical oxidation is caused by visible light, because most of the ultraviolet light would have been absorbed by the double thickness of Pyrex glass in the illumination apparatus shown in Fig. 2. Furthermore, when a test solution in a quartz flask was exposed to the radiations of a mercury vapor ultraviolet light, with visible light filter, no marked photochemical development of color occurred. It is recognized, however, that the destructive effects of illumination may have been caused by low-intensity ultraviolet light which may pass through Pyrex glass and water.

Whether DAB was illuminated fifteen or sixty minutes, the photochemical after effect, in four hours, produced pre-nitrite color equivalent to 55 to 58% of the total potentially available color. This proportion of the color was probably not determined by the availability of dissolved oxygen because in a test in which oxygen was bubbled into the solution during the fifteen minute illumination period the same amount of pre-nitrite color developed as when illumination was conducted in a closed tube. These results suggested the possibility that the after effect might be caused by a trace of contaminant in the DAB which was the limiting factor in determination of the magnitude of the after effect. To test this hypothesis, tests containing 10, 30, 60 and 100 mg. of DAB per test were illuminated fifteen minutes each and the after effect produced in twenty-four hours was determined. Pre-nitrite colors representing 58, 72, 72 and 72% of the total, respectively, were obtained. If the after effect were dependent on a contaminant in the DAB the proportion of pre-ni-

(9) Cole, *J. Physiol.*, **30**, 311 (1903–1904).

(10) Bäckström's work has been discussed in detail by Kistiakowsky and Dhar, ref. 8.

trite color should have increased in proportion to the quantity of DAB used per test. This did not occur. Therefore it was concluded that the after effect was caused by DAB and not by a contaminant.

Illumination during the first five minutes of reaction I caused a loss of 25% of the tryptophan, but illumination of free tryptophan for thirty minutes and illumination of the final condensation product of tryptophan and DAB for 120 minutes caused only 3 and 6% losses, respectively. These observations indicate that an intermediate compound in the condensation of tryptophan and DAB is more susceptible to destruction by light than is either free tryptophan or the condensation product. The importance of protecting test solutions in analytical procedures from light particularly during the first five minutes of reaction I is therefore obvious. It is also important to protect acid solutions of DAB from light because loss of as much as 10% of the tryptophan may result from using illuminated DAB solutions.

Experimental

General procedure, apparatus and materials used in this study have been described in detail in a previous paper.[2] Transmittancies were determined with a Coleman spectrophotometer, Model 11, excepting the curves in Fig. 1 which were obtained with a Beckman quartz spectrophotometer. New procedures or variations from procedures previously described[2] are given in footnotes in the tables.

Reagent grade sulfuric acid was distilled using all glass joints. The first 8% of the distillate was discarded and a fraction representing about 85% boiling at 327 to 332° (uncor.) was collected in a receiver closed with a calcium chloride tube. This acid was used to prepare the sulfuric acid solutions used for experiments described in Tables III and IV as well as for some of the other tests.

Light Source and Illumination Procedure.—The light source was a 115-volt, No. 2 Photoflood, Mazda lamp made by General Electric Co. Lamps were mounted in a nine-inch aluminum coated reflector. The procedure for illumination was standardized as follows. The solution to be illuminated was placed in Tube A, Fig. 2. Tube A was placed in the glass holder B through which water at 25 + 0.1° was circulated at a rate of 5 liters per minute. Under these conditions the rise in temperature of the solution in Tube A was no more than 0.1 to 0.2° regardless of the length of time of illumination. The solution was illuminated with the outer tip of the light bulb 9 inches from the center of B so that the rays of light fell perpendicularly on the side of Tube A. The intensity of Photoflood lamps gradually declines with use. To minimize this effect fresh lights were used for short periods and used bulbs for longer periods of illumination.

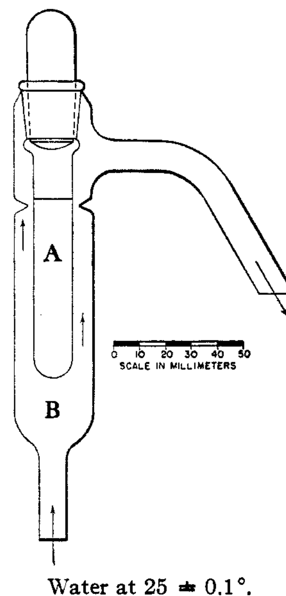

Fig. 2.—Illumination apparatus.

Summary

The photochemistry of tryptophan, *p*-dimethylaminobenzaldehyde and their reaction products in sulfuric acid has been studied. Evidence indicates that an intermediate compound formed in the condensation of tryptophan and *p*-dimethylaminobenzaldehyde is more susceptible to destruction by light than is either free tryptophan or the final condensation product. The photochemical development of color is caused by a visible portion of the spectrum and not by ultraviolet light. Previously illuminated acid solutions of *p*-dimethylaminobenzaldehyde cause the development of color and also some destruction of tryptophan as after effect when tryptophan is subsequently added to such solutions. Probable mechanism of the photochemical reactions is discussed.

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