supplying the culture of the test fungus to carry out the fungicidal work in this Laboratory and lastly to the Board of Scientific and Industrial Research, Government of Orissa, for a research grant.

CUTTACK-3, INDIA

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

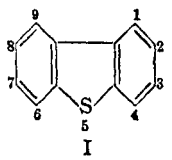
Nitration of 1-Hydroxythianthrene and of 1-Hydroxythianthrene-5,10-tetroxide

BY HENRY GILMAN AND DHAIRYASHEEL R. SWAYAMPATI

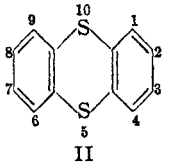
RECEIVED SEPTEMBER 24, 1956

1-Hydroxythianthrene was prepared in greatly improved yield by a modification of an earlier procedure. The treatment of the phenol with nitric acid (sp. gr. 1.2) in glacial acetic acid at reflux temperature resulted in the formation of 2,4-dinitro-1-hydroxythianthrene-5(or 10)-oxide in 61–65% yield. Reduction of the sulfoxide with hydrogen bromide gave 2,4-dinitro-1-hydroxythianthrene from which the same sulfoxide was regenerated by oxidation with the nitric acid. 1-Hydroxythianthrene-5,10-tetroxide reacted with nitric acid (sp. gr. 1.5) to give a fair yield of 2,4-dinitro-1-hydroxythianthrene-5,10-tetroxide.

Dibenzothiophene (I) can be nitrated with nitric acid to give a mixture of 2-nitrodibenzothiophene and dibenzothiophene-5-oxide.[1] The sulfoxide, dibenzothiophene-5-oxide, can also be nitrated to yield 3-nitrodibenzothiophene-5-oxide.[2] This capacity of dibenzothiophene and dibenzothiophene-5-oxide of being nitrated is not shared by thianthrene (II) or any of its oxides.



I



II

Treatment of thianthrene with dilute nitric acid (sp. gr. 1.2) in glacial acetic acid at reflux temperature results in the formation of thianthrene-5-oxide in very good yields.[3–8] Nitric acid has also been used in the preparation of thianthrene-5,10-dioxide,[5,7] thianthrene-5,5,10-trioxide[4,7] and thianthrene-5,10-tetroxide.[8–11] Nitric acid (sp. gr. 1.5) oxidizes II to the tetroxide.[3,7,8,12] If II is dissolved in fuming nitric acid and the solution repeatedly evaporated to dryness, a quantitative yield of thianthrene-5,10-tetroxide is obtained. The action of a mixture of nitric and sulfuric acids does not proceed beyond oxidation of II to the tetroxide.[13] Even where a number of methoxyl[5,7] or methyl[6,7,9,10,12] groups are present, the treatment with nitric acid proceeds in the same manner as with the parent substance II.

The present investigation deals with the nitration of thianthrene and some of its derivatives. Our attempts of nitrating II by dissolving it in nitric acid (sp. gr. 1.5) at −10° and at −30° were unsuccessful and resulted in the formation of the α-form[14] of thianthrene-5,10-dioxide. Subsequently, 1-hydroxythianthrene (III) was selected as a promising derivative for nitration in the thianthrene system. This phenol has been prepared[15] by metalating thianthrene with *n*-butyllithium and oxidizing the thianthrenyllithium with oxygen in the presence of *n*-butylmagnesium bromide. The yield of the phenol, however, was only 2.5%. By suitably modifying the procedure, particularly in the working up of the reaction mixture, we were able to obtain the product in 46% yield. 1-Thianthreneoxyacetic acid and its ethyl ester were prepared as derivatives which may have activity as plant hormones.

The presence of the hydroxyl group was found to make the thianthrene molecule very susceptible to nitration. In an attempt of preparing a monosulfoxide of III by the usual treatment with the dilute nitric acid in glacial acetic acid at reflux temperature for 30 minutes, we obtained, instead, a dinitro-1-hydroxythianthrene-5(or 10)-oxide (IV) in 61% yield. Reduction of IV with hydrogen bromide gave a dinitro-1-hydroxythianthrene (V), which upon treatment with the dilute nitric acid in glacial acetic acid at reflux temperature for 15 minutes gave a product which was identical with IV. The infrared spectrum of V showed, among others, an absorption band at 13.4μ, characteristic of 1,2-substitution, which meant that one of the two benzene rings was only disubstituted. The dinitration, therefore, occurred in the same benzene ring which carried the hydroxyl group. The two nitro groups must have been substituted in the 2,4- positions in view of the powerful *ortho–para* directing influence of the phenolic hydroxyl group in electrophilic reactions. Hence the structure of V must be 2,4-dinitro-1-hydroxythianthrene and that of IV, 2,4-dinitro-1-hydroxythianthrene-5(or 10)-oxide. Whether the sulfoxide group in IV was in the 5- or in the 10-position could not be established with any amount of certainty. The conversion of V to IV, upon oxidation, is interesting in view of the fact that an isomer of IV in which the oxygen atom of the sulfoxide group is on the sulfur atom different from that in IV was equally pos-

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(11) M. Tomita, *J. Pharm. Soc. Japan*, **58**, 517 (in German, 139) (1938) [*C. A.*, **32**, 7463 (1938)].

(12) J. B. Cohen and F. W. Skirrow, *J. Chem. Soc.*, **75**, 887 (1899).

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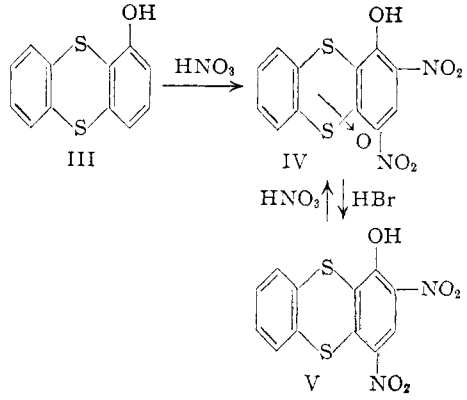
(15) H. Gilman and D. L. Esmay, THIS JOURNAL, **76**, 5787 (1954).

sible. This means that the possibility of the oxidation following nitration in the formation of IV from III is not ruled out. If a sulfoxide other than IV had been obtained from the oxidation of V with the dilute nitric acid, it would have been established that nitration did not precede oxidation.

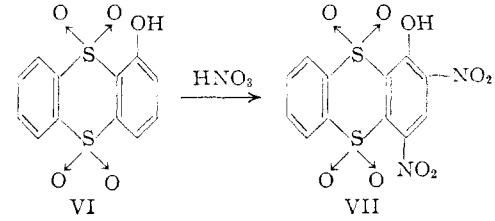


In order to determine whether the oxidation took place before, after or simultaneously with nitration in the formation of IV from III, an attempt was made to arrest the reaction at an intermediate stage. When the total time of the reaction of III with the dilute nitric acid in glacial acetic acid at reflux temperature was limited to 1 minute, IV was still obtained in comparable yield (65%). It appears from this that the two processes, oxidation and nitration, probably take place simultaneously.

The oxidation of 1-hydroxythianthrene to 1-hydroxythianthrene-5,10-tetroxide (VI) greatly decreased the ease of nitration of the phenol. Treatment of VI with the dilute nitric acid in glacial acetic acid at reflux temperature for 30 minutes failed to nitrate it, and the starting material was recovered in 90% yield. However, nitration with nitric acid (sp. gr. 1.5) gave a fair yield of a dinitro-1-hydroxythianthrene-5,10-tetroxide (VII). Since thianthrene-5,10-tetroxide itself is unreactive to nitration,[12,13] the dinitration of VI must have been possible only due to the presence of the hydroxyl group in the molecule. The effect of the phenolic hydroxyl group is thus much greater than that of the sulfone groups in the nitration process. Again, in view of the powerful *ortho–para* directing influence of the hydroxyl group, the two nitro groups must have entered the 2,4-positions. The structure of VII must therefore be 2,4-dinitro-1-hydroxythianthrene-5,10-tetroxide. It was believed that the oxidation of IV with hydrogen peroxide should give VII. However, the product obtained gave analyses corresponding to a 2,4-dinitro-1-hydroxythianthrene-trioxide. The oxidation probably did not proceed to the tetroxide stage due to the steric hindrance of the nitro group in the 4-position. The trioxide may therefore be 2,4-dinitro-1-hydroxythianthrene-5,10,10-trioxide.

1-Methoxythianthrene was obtained in good yield by methylating 1-hydroxythianthrene with dimethyl sulfate. No pure product was obtained from the nitration of the methoxythianthrene.

The 2,4-dinitro-1-hydroxythianthrene had a beautiful, golden-orange color which was progressively destroyed upon oxidation. Thus, IV was orange and VII had only a slight yellow color.



Experimental[16]

1-Hydroxythianthrene (III).—To a stirred suspension of 129.6 g. (0.6 mole) of thianthrene (II)[17] in a liter of anhydrous ether was added 685 ml. (0.72 mole) of *n*-butyllithium,[18] and the mixture was stirred at room temperature for 30 hours. At the end of this period Color Test I[19] was positive and Color Test II[20] was only slightly positive.

To the stirred suspension was added slowly an ethereal solution of 0.72 mole of *n*-butylmagnesium bromide[21] in 500 ml. of anhydrous ether. The reaction flask was immersed in an ice-bath during the addition of the *n*-butylmagnesium bromide solution. A clear, steel-gray solution resulted which was subsequently stirred vigorously while oxygen was allowed to sweep over the surface at a gentle rate. The rate of oxygen was adjusted so as to maintain a gentle reflux of the ether. Color Test I, examined after 12 hours, was found to be negative. The oxygen supply was cut off, the reaction flask immersed in an ice-bath and cold 10% hydrochloric acid was added, while stirring, until the aqueous layer was acidic to congo red. The layers were separated and the ethereal layer was extracted with two 300-ml. portions of cold 10% potassium hydroxide solution.

The combined extract was warmed to expel the dissolved ether, treated with Norit A, filtered, cooled and acidified with 10% hydrochloric acid. The sirupy, dark liquid which separated hardened to a brown solid upon standing for two days. The crude product was dried over phosphorus pentoxide in a vacuum desiccator and subsequently distilled under reduced pressure. The fraction, b.p. 169–170° (0.2 mm.), solidified to a white product which was nearly pure 1-hydroxythianthrene, melting at 113–115°. The pure 1-hydroxythianthrene melts at 117–118°.[15] The yield of the product was 48.0 g. (46%). A second fraction hardened to a pink solid, 6.8 g., melting over the range 108–117°, which must be impure III.

1-Hydroxythianthrene-5,10-tetroxide (VI).—To a hot solution of 4.64 g. (0.02 mole) of III in 30 ml. of glacial acetic acid was added a solution of 13 g. of 30% hydrogen peroxide in 13 ml. of glacial acetic acid. A slight yellow color developed in the solution. A clear, colorless solution was obtained during a reflux period of 2 hours. A white product which crystallized out upon cooling was filtered, washed and dried to yield 4.54 g. of VI, melting at 239–240°. An additional 0.3 g. of VI was obtained upon dilution of the mother liquor. The total yield of VI was 4.84 g. (82%).

Anal. Calcd. for $C_{12}H_8O_6S_2$: S, 21.62. Found: S, 21.49.

1-Hydroxythianthrene and Nitric Acid. Run I.—To a refluxing solution of 5.1 g. (0.022 mole) of III in 50 ml. of glacial acetic acid was added dropwise, over a period of 30 minutes, 10 ml. of nitric acid (sp. gr. 1.2). The solution turned dark-brown at first and subsequently red toward the end of the reaction period. The mixture was refluxed for an additional period of 15 minutes and cooled. The orange product was filtered and dried to give 4.9 g. of crude 2,4-dinitro-1-hydroxythianthrene-5(or 10)-oxide (IV) decomposing at 170°. Recrystallization from glacial acetic acid (Norit A) yielded 3.8 g. of the pure IV melting at 192° dec. From the mother liquor was obtained, upon concentration

(16) All melting points reported herein are uncorrected. Reactions involving organometallic compounds were carried out in an atmosphere of dry, oxygen-free nitrogen.

(17) K. Fleischer and J. Stemmer, *Ann.*, **422**, 265 (1921).

(18) H. Gilman, J. A. Beel, C. G. Brannen, M. W. Bullock, G. E. Dunn and L. S. Miller, THIS JOURNAL, **71**, 1499 (1949).

(19) H. Gilman and F. Schulze, *ibid.*, **47**, 2002 (1925).

(20) H. Gilman and J. Swiss, *ibid.*, **62**, 1847 (1940).

(21) E. E. Dreger, "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 306.

and cooling, another 0.7 g. of the pure IV, melting at 192° dec. The total yield of the pure, orange IV was 4.5 g. (61%). The product gave a positive test for the nitro group.

Anal. Calcd. for $C_{12}H_6N_2O_6S_2$: N, 8.28; S, 19.06. Found: N, 8.28, 8.18; S, 19.03, 19.21.

Run II.—To a solution of 0.23 g. (0.001 mole) of III in 2 ml. of boiling glacial acetic acid was added 1 ml. of nitric acid (sp. gr. 1.2). The mixture was boiled for 1 minute and immediately poured over 50 g. of ice. The orange product was filtered and dried to yield 0.22 g. (65%) of nearly pure IV, melting at 188–189° dec. A mixture of the product and that obtained from run I melted undepressed and the infrared absorption spectra of the two were identical.

2,4-Dinitro-1-hydroxythianthrene (V).—Hydrogen bromide was bubbled for 1 hour through a solution of 1.01 g. (0.003 mole) of IV in 300 ml. of glacial acetic acid. The product was dissolved in the acetic acid by warming and was cooled to 50° before commencing the addition of the hydrogen bromide. An orange product separated from the solution. The mixture was treated with excess of water, and the product was filtered, washed and dried to yield 0.90 g. (93%) of nearly pure V melting at 228–229°. Recrystallization from glacial acetic acid gave a total of 0.80 g. (83%) of pure V melting at 234°.

Anal. Calcd. for $C_{12}H_6N_2O_5S_2$: S, 19.88; N, 8.70. Found: S, 19.99; N, 8.58, 8.50.

2,4-Dinitro-1-hydroxythianthrene and Nitric Acid.—To a hot solution of 0.13 g. (0.0004 mole) of V in 20 ml. of glacial acetic acid was added 1 ml. of nitric acid (sp. gr. 1.2) and the mixture was refluxed for 15 minutes. The solution was diluted with water, and the product which separated was filtered, washed and dried to give 0.11 g. (81%) of IV, identified by its mixed melting point.

2,4-Dinitro-1-hydroxythianthrene-5(or 10)-oxide and Hydrogen Peroxide.—To a hot solution of 0.68 g. (0.002 mole) of IV in 50 ml. of glacial acetic acid was added a solution of 2 ml. of 30% hydrogen peroxide in 5 ml. of glacial acetic acid, and the resulting yellow solution was refluxed for 3 hours. No product separated upon cooling or diluting with excess water. The solution was concentrated to a volume of 5 ml., and cooled. The yellow product which separated was filtered, washed and dried to yield 0.30 g. of crude material melting over the range 190–200° dec. Recrystallization from a mixture of glacial acetic acid and ethanol yielded 0.22 g. (30%) of a trioxide of V melting at 209–210° dec. The oxidation of IV would be expected to take place at the 10-position preferentially in view of the steric hindrance of the nitro group in the 4-position. The structure of the trioxide is therefore considered to be 2,4-dinitro-1-hydroxythianthrene-5,10,10-trioxide.

Anal. Calcd. for $C_{12}H_6N_2O_8S_2$: N, 7.54. Found: N, 7.53, 7.40.

1-Hydroxythianthrene-5,10-tetroxide and Nitric Acid. Run I.—A solution of 0.30 g. (0.001 mole) of VI in a mixture of 3 ml. of glacial acetic acid and 1 ml. of nitric acid (sp. gr. 1.2) was refluxed for 15 minutes. The white product which separated upon cooling was filtered, washed and dried to give 0.16 g. of the starting material VI, identified by its mixed melting point. The evaporation of the mother liquor yielded another 0.11 g. of VI (mixed m.p.). The total recovery of the starting material was 0.27 g. (90%).

Run II.—A solution of 0.59 g. (0.002 mole) of VI in 5 ml. of nitric acid (sp. gr. 1.4) was evaporated to dryness on the steam-plate. The procedure was repeated twice and the resulting yellow product was crystallized from nitric acid (sp. gr. 1.4) to give 0.30 g. (39%) of slightly yellow crystals of VII melting at 287° dec.

Anal. Calcd. for $C_{12}H_6N_2O_9S_2$: N, 7.25. Found: N, 7.23, 7.14.

1-Methoxythianthrene.—To a stirred suspension of 34.8 g. (0.15 mole) of III in 60 ml. of water containing 6.5 g. of sodium hydroxide was added, over a period of 30 minutes, 20.8 g. (0.165 mole) of dimethyl sulfate. The temperature of the solution was maintained at 10° during the addition of the dimethyl sulfate. The suspension was stirred at room temperature for 15 minutes and subsequently refluxed for a period of 2 hours. A clear solution resulted and a white solid was deposited upon cooling. The product was filtered, washed and dried between folds of a filter paper. Crystallization from glacial acetic acid, followed by drying over phosphorus pentoxide in a vacuum desiccator gave 27.0 g. of nearly pure 1-methoxythianthrene, melting at 79–80°. Recrystallization from ethanol, followed by drying in the manner described above, gave 25.1 g. (67%) of the pure product melting at 81–81.5°.

Anal. Calcd. for $C_{13}H_{10}OS_2$: S, 26.02. Found: S, 25.93.

1-Methoxythianthrene-5,10-tetroxide.—To a hot solution of 2.46 g. (0.01 mole) of 1-methoxythianthrene in 25 ml. of glacial acetic acid was added a solution of 6.5 g. of 30% hydrogen peroxide in 5 ml. of glacial acetic acid, and the resulting solution was refluxed for a period of 2 hours. The mixture was cooled in an ice-bath and the white product which had separated was filtered and dried to yield 2.81 g. of 1-methoxythianthrene-5,10-tetroxide melting at 246–247°. Dilution of the mother liquor yielded another 0.13 g. of the pure product. The total yield of the pure compound was 2.94 g. (98%).

Anal. Calcd. for $C_{13}H_{10}O_5S_2$: S, 20.65. Found: S, 20.55.

1-Methoxythianthrene and Nitric Acid.—To a hot solution of 2.46 g. (0.01 mole) of 1-methoxythianthrene in 25 ml. of glacial acetic acid was added dropwise 5 ml. of nitric acid (sp. gr. 1.2) and the yellow solution was refluxed for a period of 15 minutes. No product separated upon cooling. The solution was concentrated to a small volume and cooled, but no product crystallized out. The solvent was removed in a current of dry air. A yellow oily residue was left. Attempts at isolating a pure product from this were unsuccessful.

1-Thianthreneoxyacetic Acid.—To a solution of 4.53 g. (0.113 mole) of sodium hydroxide in 60 ml. of water were added 11.6 g. (0.05 mole) of III and 5.92 g. (0.063 mole) of chloroacetic acid. The mixture was refluxed for 13 hours, and subsequently diluted with water and filtered. The filtrate was acidified with 10% hydrochloric acid. The cream-colored product was filtered and washed. The crude, dried material weighed 13.3 g. and melted over the range 145–155°. After several recrystallizations from 90% acetic acid there was obtained 4.3 g. (30%) of pure 1-thianthreneoxyacetic acid melting at 174–175°.

Anal. Calcd. for $C_{14}H_{10}O_3S_2$: neut. equiv., 290; S, 22.07. Found: neut. equiv., 288, 287; S, 22.57, 22.61.

Ethyl 1-Thianthreneoxyacetate.—To a solution of 0.92 g. (0.04 g. atom) of sodium in 50 ml. of absolute ethanol were added 6.68 g. (0.04 mole) of ethyl bromoacetate and 9.28 g. (0.04 mole) of III. The mixture was refluxed for 2 hours, and the resulting red solution was diluted with excess water. The mixture was extracted with ether and the extract was dried over anhydrous sodium sulfate. Evaporation of the solvent left an oil which hardened upon standing. Several recrystallizations of the crude product from methanol yielded 3.0 g. (23.6%) of pure ethyl 1-thianthreneoxyacetate melting at 90–90.5°.

Anal. Calcd. for $C_{16}H_{14}O_3S_2$: S, 20.1. Found: S, 20.2, 19.8.

1-Hydroxythianthrene and *p*-Bromobenzenediazonium Chloride.—To a hot mixture of 3.46 g. (0.02 mole) of *p*-bromoaniline in 2 ml. of water was added cautiously 4 ml. of hydrochloric acid and the hydrochloride was precipitated as fine needles by chilling. To the stirred mixture was added slowly a solution of 1.4 g. (0.02 mole) of sodium nitrite in 3 ml. of water, keeping the temperature at 0–5°. The low temperature was maintained for an additional period of 30 minutes after which the solution was filtered into a stirred solution of 4.64 g. (0.02 mole) of III in 25 ml. of water containing 2.4 g. (0.06 mole) of sodium hydroxide and 5 ml. of ethanol. The flask was immersed in an ice-bath during the addition of the diazonium salt solution and for a subsequent period of 2 hours. The bright, scarlet dye was filtered and subsequently suspended in warm water, and hydrochloric acid was added until the suspension was acidic. The dye was collected by filtration, washed and dried to yield 7.56 g. (91%) of crude 4-(4′-bromobenzeneazo)-1-hydroxythianthrene melting over the range 180–192°. Recrystallization from benzene (Norit A) yielded 5.5 g. (66%) of nearly pure dye melting at 195–196°. The analytical sample, obtained by an additional recrystallization from benzene, melted at 199–200°.

Anal. Calcd. for $C_{18}H_{11}BrN_2OS_2$: Br, 19.28; N, 6.75. Found: Br, 18.51, 18.79; N, 6.63, 6.52.

Thianthrene (II) and Nitric Acid. Run I.—To 25 ml. of nitric acid (sp. gr. 1.5) was added at −10°, 4.32 g. (0.02 mole) of powdered II. The temperature was maintained at −15° to −10° during the addition of II. The solution was immediately poured over ice and the white product which separated was filtered, washed with water and dried. Recrystallization from glacial acetic acid gave 2.23 g. (45%) of the α-form[14] of thianthrene-5,10-dioxide (mixed m.p.). The mother liquor was colorless indicating that no nitration had taken place.

Run II.—To 100 ml. of nitric acid (sp. gr. 1.5) was added at −30°, 10.8 g. (0.05 mole) of powdered II. The mixture was vigorously stirred during the addition of II and the temperature was maintained at −30° for a total period of 1 hour. Upon the addition of each portion of II to the nitric acid an intense red coloration was produced which immediately disappeared to give a greenish solution. A brown slurry was formed toward the end of the addition-period of 30 minutes. The mixture was poured over ice and the white product which separated was filtered, washed and dried to yield 4.7 g. (38%) of the α-form of thianthrene-5,10-dioxide (mixed m.p.).

Acknowledgment.—The authors wish to thank the Institute for Atomic Research, Iowa State College, for making available to us the Baird double beam infrared spectrophotometer used in the determination of the spectra of the compounds reported in this paper. We are grateful to Mr. Robert McCord for the actual determination of the spectra.

AMES, IOWA

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY RESEARCH, ROSWELL PARK MEMORIAL INSTITUTE; FROM THE SECTION OF IMMUNOCHEMISTRY OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH; AND FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY][1]

The Hydration of the Annular Nitrogen Group as a Factor in the Combination of Hapten with Antibody[2,3]

BY DAVID PRESSMAN AND MALCOLM SIEGEL

RECEIVED AUGUST 30, 1956

The relative extent of combination of each of the various pyridine carboxylate and quinoline carboxylate ions with antibody specific to the *o*-, *m*- and *p*-azobenzoate ions was determined. In each case the ion behaved like either a benzoate or napthoate with a large steric substituent in the position occupied by the ring nitrogen. This was interpreted as being due to water of hydration which was attached to the nitrogen affecting its steric configuration, thereby altering its ability to react with a site formed against a substance of known configuration. The results obtained here indicate that water of hydration may be a structural feature of significance in biological systems in general.

Most biological reactions take place in aqueous media and the hydration of the molecules involved is an important structural feature having a role in determining binding strength and specificity. Immuno-chemical systems are very useful in demonstrating that hydration is a structural feature of considerable importance.

Evidence that the hydration of the annular nitrogen group of the pyridine or pyrazine nucleus appears to be an important factor in the combination of hapten with antibody was first observed in a study of the extent of combination of various substances with antibody homologous to the 4-azophthalate ion.[4,5] The combining power of this antibody with simple substances varied markedly in the sequence phthalate ion > pyridine-2,3-dicarboxylate ion > pyrazine-2,3-dicarboxylate ion. This order of decrease was attributed to the hydration of the annular nitrogen atom. The water of hydration attached to the hapten acts sterically to interfere with the combination of these haptens with the antibody to the 4-azophthalate ion. The interference was less with the pyridine compound, which has a single nitrogen, than with the pyrazine derivative which has two nitrogen atoms.

The role of hydration has now been studied further through an investigation of the extent of combination of antiserum specific to the *o*-, *m*- and *p*-azobenzoate ion groups (anti-X_o, anti-X_m, anti-X_p antibodies, respectively) with the various pyridine monocarboxylate ions, picolinate, nicotinate and isonicotinate, and with the various quinoline monocarboxylate ions. The results are reported here.

Landsteiner and van der Scheer[6] have already reported a study of combination of nicotinate and picolinate with the three azobenzoate sera, but their studies were too qualitative and restricted to permit conclusions to be drawn such as those made here.

Experimental Methods

Materials.—The quinoline-carboxylic acids, which were kindly furnished by Prof. Robert C. Elderfield, were pure samples, recrystallized to constant melting point and the correct neutral equivalent. Heterocyclic acids and other haptens were commercial preparations recrystallized to the correct melting point and neutral equivalent.

Protein Antigens.—Immunizing antigen was prepared by diazotizing 500 mg. of *o*-, *m*- and *p*-aminobenzoic acid and coupling each with 100 ml. of regenerated lyophilized bovine serum. (The coupling mixtures were allowed to stand overnight at 3–5° and at *p*H 10.5.) The azoproteins were dialyzed against many portions of saline solution containing sodium borate until the dialyzate was free of color. Phenol was added to a concentration of 0.2%, and the solutions were stored in the cold.

Test antigens were prepared by diazotizing and coupling 26 mg. of the *o*-, *m*- and *p*-aminobenzoic acids with 250 mg. of ovalbumin at *p*H 9. After standing overnight in the cold, the coupling mixtures were dialyzed against borate solution until no further passage of color through the membrane was detected. The azoproteins were precipitated twice with acid at *p*H 3.5 and redissolved in alkaline saline with a final adjustment to *p*H 7 to 8.

(1) Contribution from the Gates and Crellin Laboratories No. 2133.

(2) Presented in part before the Division of Biological Chemistry at the 115th National Meeting of the American Chemical Society, San Francisco, 1949.

(3) This research was supported at various times by a grant from the Rockefeller Foundation, the American Cancer Society (during tenure of D. P. as Senior Fellow), and the Atomic Energy Commission, Contract No. AT(30-1)-910.

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