Amino- and Nitrofluorescein Derivatives

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Synthesis of 5- and 6-aminofluoresceins, by the reduction of the corresponding nitrofluoresceins, has been improved so that both isomers can be obtained in a highly pure state. Phase studies indicate that the aminofluoresceins form solid solutions on equilibration with aqueous hydrochloric acid. The hydrochlorides of 5- and 6-nitrofluorescein have been prepared.

The fluorescent antibody technique for the specific staining of antigens has found ever-increasing application since its introduction and development.[1-3] A recent survey demonstrated a wide variation in the composition of the aminofluoresceins and fluorescein isothiocyanates commercially available for use in this technique.[4] A reinvestigation of the procedures for synthesizing these compounds was undertaken in an attempt to obtain pure compounds and simplify the synthesis procedure.

The condensation of 4-nitrophthalic acid with resorcinol gives a mixture of 5- and 6-nitrofluorescein. This condensation was first reported by Bogert and Wright.[5] The separation and purification of the two isomers was reported by Coons and Kaplan.[2] Since it has never been established which product is the 5- and which is the 6-substituted fluorescein, they will be referred to as nitro- and aminofluorescein I and II, according to the Coons and Kaplan designation.

In this laboratory, the preparation and separation of nitrofluorescein I and II was carried out without difficulty according to the method of Coons and Kaplan. However, considerable difficulty was encountered in attempting to reduce the nitrofluoresceins to the corresponding aminofluoresceins by the use of hydrogen and Raney nickel. The difficulty encountered in preparing Raney nickel with exactly reproducible activity may be the reason for the variation in composition of the commercially available aminofluoresceins.

Since reduction methods requiring an acidic medium, such as stannous chloride and hydrochloric acid and various metal and hydrochloric acid combinations, have not been found to be satisfactory,[1,5] it was decided to investigate an alkaline reduction system using the sulfide or hydrosulfide ion. Ammonium sulfide and sodium hydrosulfide have both been used successfully for the reduction of *m*-dinitrobenzene to *m*-nitroaniline.[6-8]

Although neither sodium sulfide nor sodium hydrosulfide alone gave satisfactory reduction of the nitrofluoresceins, it was found that an aqueous solution of the two together gave complete reduction to the amines with no detectable side reactions. The method was simple and reliable, reduction being carried out on as much as 40 grams of nitrofluorescein in one batch. Purity of the two aminofluorescein isomers was confirmed by elemental analysis. Coons and Kaplan reported the elemental analysis of the monohydrochlorides of the two aminofluorescein isomers but not of the free amines.

The present workers were unsuccessful in repeating the preparation of the hydrochlorides of aminofluorescein I and II by recrystallization from 2 *N* hydrochloric acid, as reported by Coons and Kaplan. It was observed as the latter authors reported, that aminofluorescein II did tend to form rosettes of red colored crystals, but it was found that these crystals did not consistently have a molar ratio of hydrogen chloride to aminofluorescein of one. Repeated efforts to prepare the monohydrochlorides resulted in products with varying hydrogen chloride content, depending on the rate of cooling and the length of time the crystals were allowed to remain in contact with the supernatant solution.

In view of this inconsistency, a phase study was undertaken in an attempt to find a range of hydrochloric acid concentrations from which the monohydrochlorides could be obtained. The results of this study are presented in Tables I and II.

It is evident, from Tables I and II, that a product with a hydrogen chloride to aminofluorescein ratio of one cannot be obtained by equilibration with a very wide range of hydrochloric acid concentrations. The data in these tables would indicate that neither isomer I nor isomer II of the aminofluoresceins form stable hydrochlorides. The red crystals of monohydrochloride obtained by Coons and Kaplan were probably metastable products, whereas solid solutions with water and hydrochloric acid appear to be formed under conditions of true equilibrium.

As shown by Tables I and II, the hydrogen chloride to aminofluorescein ratios for the solid phase were greater than one where the concentra-

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(5) M. T. Bogert and R. G. Wright, *J. Am. Chem. Soc.*, **27**, 1310 (1905).

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(7) K. Brand, *J. prakt. Chem.*, [2] **74**, 463 (1906).

(8) H. H. Hodgson and E. R. Ward, *J. Chem. Soc.*, 1316 (1949).

TABLE I

EQUILIBRATION OF AMINOFLUORESCEIN I WITH HYDROCHLORIC ACID

Initial *N* HCl used[a]	Liquid phase at equilibrium: % HCl	Liquid phase at equilibrium: % Aminofluorescein I	Solid phase at equilibrium: Mole fraction HCl	Solid phase at equilibrium: Mole fraction aminofluorescein I	Solid phase at equilibrium: Mole fraction HCl / Mole fraction aminofluorescein I
0.130	0.453	0.0445	0.459	0.541	0.848
.160	.529	.0438	.048	.520	0.922
.340	1.21	.0555	.512	.488	1.05
.500	1.80	.0594	.531	.469	1.13
.700	2.52	.0928	.574	.426	1.35
1.70	6.17	.184	.617	.383	1.61

[a] In each case 500 mg. of aminofluorescein I was added to 250 ml. of hydrochloric acid of the indicated normality.

TABLE II

EQUILIBRATION OF AMINOFLUORESCEIN II WITH HYDROCHLORIC ACID

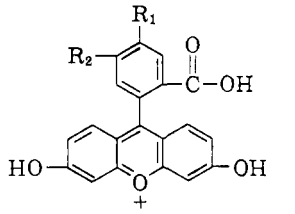
Initial *N* HCl used	Liquid phase at equilibrium: % HCl	Liquid phase at equilibrium: % Aminofluorescein II	Solid phase at equilibrium: Mole Fraction HCl	Solid phase at equilibrium: Mole fraction aminofluorescein II	Solid phase at equilibrium: Mole fraction HCl / Mole fraction aminofluorescein II
0.500[a]	1.74	0.0896	0.431	0.569	0.758
0.700[a]	2.47	0.0858	0.543	0.457	1.19
1.70[b]	5.88	0.261	0.610	0.390	1.56

[a] In this case 400 mg. of aminofluorescein II was added to 50 ml. of hydrochloric acid. [b] An 800-mg. sample of aminofluorescein II was added to 50 ml. of hydrochloric acid.

tion of hydrochloric acid in the liquid phase was high. This probably is due to oxonium ion formation in addition to formation of the amine hydrochloride. Gattermann[9] and Orndorff and Hemmer[10] reported the preparation of a hydrochloride of fluorescein. Although there has been no report of the nitro, amino, or isothiocyanate substituted fluoresceins forming similar oxonium salts, this would be expected.

The hydrochlorides of the two nitrofluorescein isomers were prepared by equilibrating them with concentrated hydrochloric acid. The infrared spectra of these compounds indicate that they are carboxylic acids.[11] The intense sharp absorption peaks found at 1711 cm.$^{-1}$ for nitrofluorescein I·hydrochloride and 1697 cm.$^{-1}$ for nitrofluorescein II·hydrochloride, and the broad absorption band over the region 3500–2500 cm.$^{-1}$ are evidence for the open carboxylic acid structure rather than the lactone structure attributed to free unsubstituted fluorescein.[12] The hydrochloride of fluorescein was prepared for comparison. This also showed a broad absorption band over the range 3500–2500 cm.$^{-1}$ and a strong sharp peak at 1710 cm.$^{-1}$ Although aromatic carboxylic acid carbonyl groups usually absorb in the region 1700–1680 cm.$^{-1}$, the presence of strong electron withdrawing groups on the ring would be expected to cause a shift toward the higher frequency.[13]

Considering the evidence for the presence of the free carboxylic acid group, it appears likely that the hydrochlorides of the two nitrofluorescein isomers are the oxonium salts of 5- and 6-nitrofluorescein. The additional resonance energy of this aromatic system would account for the stability of the oxonium ion.



Hydrochloride of 5-nitrofluorescein. $R_1 = NO_2$, $R_2 = H$
Hydrochloride of 6-nitrofluorescein. $R_1 = H$, $R_2 = NO_2$

Experimental

Aminofluorescein I.—To a solution of 8.7 g. of sodium sulfide nonahydrate in 150 ml. of water, 3.77 g. of nitrofluorescein I was added. When the amine was completely dissolved, 4.07 g. of sodium hydrosulfide[14] was added and the solution was refluxed for 24 hr. After cooling to room temperature, the solution was acidified with glacial acetic acid and the dark red precipitate collected on a sintered glass suction filter. This product was dissolved in 300 ml. of refluxing 6% hydrochloric acid, and filtered hot through a sintered glass filter to remove the elemental sulfur. After allowing to crystallize, the product was collected and recrystallized a second time from 100 ml. of 6% hydrochloric acid. The product from the second recrystallization was dissolved in 350 ml. of 0.5% sodium hydroxide and precipitated as the free amine by acidifying with 7 ml. of glacial acetic acid. The yield of aminofluorescein I was 2.72 g. (78%). The compound did not melt completely but underwent a slow softening and decomposition above 220° (reported[2] m.p. 215–220°, dec.).

Anal. Calcd. for $C_{20}H_{13}NO_5$: C, 69.16; H, 3.77; N, 4.03. Found: C, 69.32; H, 3.68; N, 4.01.

(9) L. Gattermann, *Ber.*, **32**, 1127 (1899).

(10) W. R. Orndorff and A. J. Hemmer, *J. Am. Chem. Soc.*, **49**, 1272 (1927).

(11) Infrared spectra were determined with a Perkin-Elmer Model 21 spectrophotometer. The potassium bromide pellet technique was used.

(12) M. Davies and R. L. Jones, *J. Chem. Soc.*, 120 (1954).

(13) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," 2nd ed., J. Wiley & Sons, Inc., New York, N. Y., 1958, p. 168.

(14) The sodium hydrosulfide was obtained from Fisher Scientific Company. It was labeled "Sodium Sulfhydrate NaHS + Aq."

Aminofluorescein II.—The reduction of 3.77 g. of nitrofluorescein II was carried out in the same manner as was isomer I, the only difference being that 150 ml. of 6% hydrochloric acid was required for the second recrystallization. There was obtained 2.17 g. (62.5%) of aminofluorescein II, m.p. 314–316°, dec. (reported[2] m.p. 315–316°, dec.).

Anal. Calcd. for $C_{20}H_{13}NO_5$: C, 69.16; H, 3.77; N, 4.03. Found: C, 69.04; H, 3.82; N, 4.03.

Phase Studies—Aminofluorescein I.—Samples of 500 mg. of aminofluorescein I were placed in 500-ml. erlenmeyer flasks to which were added 250-ml. portions of dilute hydrochloric acid covering a range in normality as shown in Table I. The samples were dissolved by heating to the boiling point under reflux. After cooling to room temperature, the flasks were sealed with glass stoppers and shaken on a mechanical shaker for about 1 week at a temperature of 20 ± 1°. In samples where the lower concentrations of hydrochloric acid were used, precipitation occurred immediately upon cooling to room temperature. Where the higher normality of hydrochloric acid was used, precipitation sometimes was not observed until the second day of equilibration. The mixtures were shaken until the concentration of aminofluorescein in solution remained constant for at least 3 days. The total time allowed for equilibration for all samples was 5–7 days.

For analysis of the liquid phase, a portion of the supernatant liquid was filtered by gravity through a sintered glass filter, and suitable aliquots were taken for determination of aminofluorescein and hydrogen chloride. Chloride was determined by potentiometric titration using 0.0500 *N* silver nitrate. Aminofluorescein was determined by diluting an aliquot of the filtrate with 0.1 *N* sodium hydroxide and measuring the absorbance at 488 mμ, the wave length of maximum absorption in the visible region.[15-17] The concentration of aminofluorescein in the aliquot was calculated from the absorbance value and an extinction coefficient determined from a range of concentrations of the aminofluorescein in 0.1 *N* sodium hydroxide.

The solid phase of each sample was collected by suction filtering through a sintered glass filter. The filter with the solid product then was placed in a vacuum desiccator, evacuated, and dried over silica gel for 48 hr. A portion of each solid sample then was placed in a weighing tube and brought to constant weight by further drying in an evacuated desiccator over silica gel at room temperature. A portion of the dried sample was dissolved in 5 ml. of 5% sodium hydroxide, acidified with concentrated nitric acid, and the chloride content determined by potentiometric titration with 0.0500 *N* silver nitrate. The aminofluorescein content was determined on another portion of the dried sample by using the method described for the filtrate.

Aminofluorescein II.—This phase study was carried out in the same manner as for isomer I, except that 400-mg. samples of aminofluorescein II and 50-ml. portions of 0.500 and 0.700 *N* hydrochloric acid were used for the equilibration. In the case where 1.7 *N* hydrochloric acid was used, 800 mg. of aminofluorescein II was added. Although the solubility of aminofluorescein II at equilibrium was not greatly different from that of isomer I, as indicated in the third column of Tables I and II, precipitation did not occur readily at the concentrations used for isomer I.

Nitrofluorescein I·Hydrochloride.—A mixture of 2.00 g. of nitrofluorescein I and 250 ml. of concentrated hydrochloric acid was shaken on a mechanical shaker for 4 hr. The solid then was collected on a sintered glass filter by suction. After drying in a vacuum desiccator over silica gel, it was powdered in an agate mortar. This procedure was repeated a second time to insure equilibration with the hydrochloric acid. It was finally shaken with concentrated hydrochloric acid, collected on the suction filter, and washed with dry dimethoxyethane that was saturated with dry hydrogen chloride. After drying to constant weight in a vacuum desiccator over silica gel, 1.32 g. (62%) of nitrofluorescein I·hydrochloride was recovered. The compound slowly lost hydrogen chloride at temperatures as low as 100°.

Anal. Calcd. for $C_{20}H_{12}ClNO_7$: C, 58.05; H, 2.92; Cl, 8.57; N, 3.39. Found: C, 57.88; H, 3.32; Cl, 8.59; N, 3.50.

Nitrofluorescein II·Hydrochloride.—The same procedure was used as for isomer I. The yield was 1.69 g. (80%). The compound slowly lost hydrogen chloride at temperatures as low as 100°.

Anal. Calcd. for $C_{20}H_{12}ClNO_7$: C, 58.05; H, 2.92; Cl, 8.57; N, 3.39. Found: C, 57.74; H, 3.09; Cl, 8.55; N, 3.33.

Fluorescein Hydrochloride.—Fluorescein hydrochloride was prepared in the same manner as the nitrofluorescein hydrochloride isomers. From 0.500 g. of fluorescein 0.488 g. of fluorescein hydrochloride was obtained (80%). The fluorescein hydrochloride slowly lost hydrogen chloride at temperatures as low as 100°.

Anal. Calcd. for $C_{20}H_{13}ClO_5$: Cl, 9.61. Found: Cl, 9.64.

(15) W. R. Orndorff, R. C. Gibbs, and C. V. Shapiro, *J. Am. Chem. Soc.*, **50**, 819 (1928).

(16) E. W. Emmart, *Arch. Biochem. Biophys.*, **73**, 1 (1958).

(17) Visible spectra were determined with a Cary recording spectrophotometer.