[CONTRIBUTION FROM THE CHEMICAL LABORATORY, HARVARD UNIVERSITY, DEPARTMENT OF ANATOMY, HARVARD MEDICAL SCHOOL, AND DEPARTMENTS OF SURGERY, SINAI HOSPITAL OF BALTIMORE AND THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE]

Preparation of Some N-(1-Naphthyl)-maleimides as Sulfhydryl Group Reagents[1a,1b]

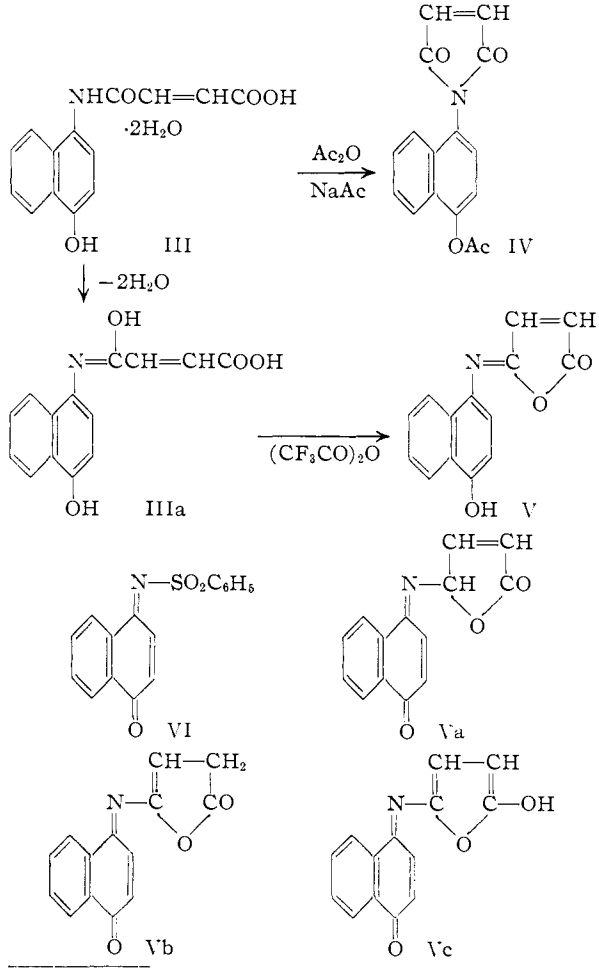
BY KWAN-CHUNG TSOU, RUSSELL J. BARRNETT AND ARNOLD M. SELIGMAN

RECEIVED FEBRUARY 21, 1955

A number of N-(1-naphthyl)-maleimides were prepared for the histochemical demonstration of protein-bound sulfhydryl groups. When N-(4-hydroxy-1-naphthyl)-maleamic acid (III) was treated with trifluoroacetic anhydride, N-(4-hydroxy-1-naphthyl)-isomaleimide (V) was obtained. This compound turned out to be an excellent reagent for the histochemical reaction. The structure of the maleimide derivatives are discussed in the light of their chemical properties, ultraviolet and infrared spectra.

Histochemical methods for the demonstration of protein-bound sulfhydryl groups have been developed in these laboratories.[2,3] Since native proteins and enzymes usually assume a rigid steric configuration,[4] the sulfhydryl groups in them may exhibit different degrees of reactivity owing to different orientation of the sulfhydryl groups within the protein. Such differences conceivably may be demonstrated by various reagents for detecting sulfhydryl groups. Therefore, although the available methods are satisfactory, the development of other methods for comparison of the results would seem worthwhile. Friedmann, Marrian and Simon-Reuss[5] have shown that N-ethylmaleimide (I) reacts with sulfhydryl groups specifically and very rapidly. In order to take advantage of these properties and explore the usefulness of such a reagent for histochemistry, a number of N-(hydroxysubstituted-1-naphthyl)-maleimides was prepared. These compounds, after reaction with protein-bound sulfhydryl groups, could be made to couple *in situ* with a suitable diazonium salt to form a protein-bound azo dye, in a manner similar to that used in other colorimetric and histochemical methods which have been developed in these laboratories.[2,6-10]

N-(4 and 5-hydroxy-substituted-1-naphthyl)-maleimides (II and XI) were prepared by fusion of a mixture of maleic anhydride, sodium bicarbonate and the aminonaphthol hydrochlorides. The yields were low, because the products tend to form a dark gum on standing in solution and therefore are difficult to purify. Attempts were also made to prepare them by cyclization of the corresponding naphthylmaleamic acids. Although the maleamic acids could be obtained in crystalline form and in good yield by the reaction of maleic anhydride and the aminonaphthol in glacial acetic acid, attempts at cyclization by either direct distillation with or without phosphorus pentoxide,[11] or by treatment with acetyl chloride failed to yield the desired product. Reaction of N-(4-hydroxy-1-naphthyl)-maleamic acid (III) with acetic anhydride and sodium acetate yielded N-(4-acetoxy-1-naphthyl)-maleimide (IV). Hydrolysis of the O-acetyl group without opening the cyclic imide ring was, however, not successful. When III was treated with trifluoroacetic anhydride, an orange-red product



(1) (a) This work was supported by grants from the National Cancer Institute (C-312), and National Institute of Arthritis and Metabolic Diseases (A-452), National Institutes of Health, Department of Health, Education, and Welfare, by an institutional grant to Harvard University from the American Cancer Society and by the Slosberg Fund for Research in Diabetes. (b) A preliminary report of this work was made at the Symposium on Glutathione, held at Ridgefield, Connecticut, November, 1953. "Glutathione," Academic Press, Inc., New York, N. Y., p. 226, 1954.

(2) R. J. Barrnett and A. M. Seligman, *Science*, **116**, 323 (1952).

(3) R. J. Barrnett and A. M. Seligman, *J. Nat. Cancer Institute*, **14**, 767 (1954).

(4) N. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

(5) E. Friedmann, D. H. Marrian and I. Simon-Reuss, *Brit. J. Pharmacol.*, **4**, 105 (1949).

(6) K. C. Tsou and A. M. Seligman, THIS JOURNAL, **74**, 3066 (1952).

(7) R. B. Cohen, K. C. Tsou, S. H. Rutenberg and A. M. Seligman, *J. Biol. Chem.*, **194**, 239 (1952).

(8) K. C. Tsou and A. M. Seligman, THIS JOURNAL, **74**, 5605 (1952).

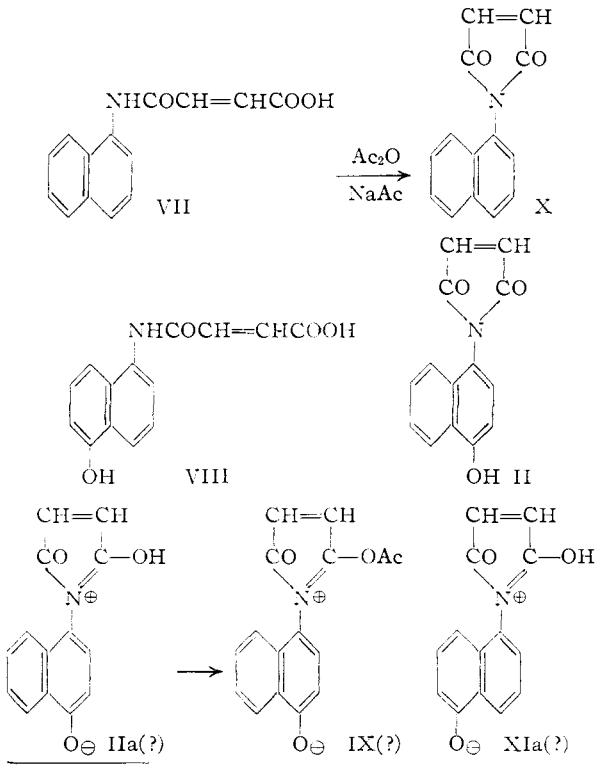
(9) A. M. Seligman, K. C. Tsou, S. H. Rutenberg and R. B. Cohen, *J. Histochem., Cytochem.*, **2**, 209 (1954).

(10) L. P. Weiss, K. C. Tsou and A. M. Seligman, *ibid.*, **2**, 29 (1954).

(11) D. H. Marrian, *J. Chem. Soc.*, 1515 (1949).

(V) was obtained. This compound gives a positive ferric chloride test (phenolic OH), couples with diazotized diorthoanisidine in sodium bicarbonate suspension but not in dilute acetic acid and when its alcoholic solution is treated with alkali, it gives a wine-red color that turns purple and then fades gradually on standing. Comparison of its ultraviolet spectrum with that of 1,4-naphthoquinone-monobenzenesulfonimide[12] (VI) excludes the possibility of Va, Vb and Vc. Furthermore, its infrared spectrum in dioxane has 3.10, 5.57 (5-lactone) and 5.99 μ (C=N) bands. The isomaleimide structure (V) therefore has been assigned.

Even though confirmatory evidence is yet to be assessed, the structure of the other compounds also can be discussed in the light of their chemical properties and their ultraviolet and infrared spectra. While N-(1-naphthyl)-maleamic acid (VII) and N-(5-hydroxy-1-naphthyl)-maleamic acid (VIII) are yellow, N-(4-hydroxy-1-naphthyl)-maleamic acid (III) is yellow as a dihydrate, but red in its anhydrous form. It seems reasonable to attribute the chromotropic difference to the possible existence of the 4-hydroxy compound in its lactim form (IIIa) which would show a deeper color due to an extended conjugated system of unsaturation. This rationalization is supported by its readiness to undergo cyclization to the isomaleimide (V). Compounds VII and VIII are not cyclizable under comparable conditions. The infrared spectrum (Table I) of compound IIIa has a conspicuous absence of the 6.10 μ band (amide) whereas in that of the other two compounds this strong amide band is present. Because of the poor solubility in the ordinarily used solvents the spectrum was made in a potassium iodide plate. The NH region of the spectrum is, therefore, obscured by the hydrogen bonding. The ultraviolet spectra of these compounds also show significant differences in that IIIa has a split maxima at 209 and 237 mμ whereas VII and VIII have a common peak at 222 mμ. If we interpret the difference in ultraviolet data of IIIa as compared to VII and VIII as due to the presence of the chromophore group —N=C(OH)—CH=CH—COOH in IIIa and the chromophore group —NHCOCH=CH—COOH in III and VII, then we could postulate N-(4-hydroxy-1-naphthyl)-maleimide (II), the ionic structure IIa on the basis of split maxima at 212 and 232 mμ and for N-(1-naphthyl)-maleimide, which does not have a split maxima, the structure X. Further evidence is provided by the fact that II does not yield IV on treatment with acetic anhydride and sodium acetate. Its instability and brilliant color changes in alkali and its high melting point suggest structure IIa. The infrared spectrum of the acetoxy derivative of II is not that of IV and apparently is an isomer of probable structure IX. Furthermore, in N-(5-hydroxy-1-naphthyl)-maleimide, the same split in maxima was observed suggesting the structure XIa.



(12) R. Adams and R. A. Wankel, THIS JOURNAL, **73**, 131 (1951).

Although the histochemical use of the compounds described here will be presented in another communication, the following results are pertinent. The sulfhydryl groups of protein could be demonstrated with N-(4-hydroxy-1-naphthyl)-maleimide (II), N-(4-hydroxy-1-naphthyl)-isomaleimide (V) and 1,4-naphthoquinone monobenzenesulfonimide (VI) as well as with the reagent developed earlier (2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD)).[2,3] These reactions were inhibited by prior treatment of the tissue sections with iodine, iodoacetate, iodoacetamide or N-ethylmaleimide (I). The reactions were reversed by treatment of the sections with an excess of glutathione. Of the four reagents the most intense staining was noted with the isomaleimide (V). The advantages of this reagent over those of DDD are that the reaction proceeded in minutes, rather than hours, and at a lower *p*H, which preserves histological structure.

Experimental[13,14]

N-(1-Naphthyl)-maleamic Acid (VII).—To 6.0 g. of maleic anhydride in 100 cc. of glacial acetic acid was added 8.4 g. of 1-naphthylamine. The reaction mixture was well-stirred and a yellow solid began to appear. At the end of one hour, the precipitation was completed by dilution with one liter of water and the product was collected by filtration, dried and recrystallized from alcohol and water to yield pure acid as fluffy yellow needles, m.p. 147–148°, yield 86% (lit.[15] m.p. 142°).

N-(1-Naphthyl)-maleimide (X).—A mixture of 1 g. of N-(1-naphthyl)-maleamic acid, 15 cc. of acetic anhydride and 0.2 g. of sodium acetate was heated for 15 minutes on a steam-bath with the exclusion of moisture and then poured slowly into 5% sodium bicarbonate solution. The precipitate was collected, and washed well with small portions of 95% ethanol and ether; 0.5 g., m.p. 115.5–116°. Recrystallization from chloroform and 95% ethanol afforded pure X as small greenish yellow granular crystals, m.p. 116–117°.

Anal. Calcd. for $C_{14}H_9NO_2$ (223.22): C, 75.32; H, 4.06; N, 6.28. Found: C, 75.04; H, 4.08; N, 6.54.

N-(4-Hydroxy-1-naphthyl)-maleamic Acid (III).—A mixture of 4-amino-1-naphthol hydrochloride (5.98 g.), sodium

(13) All melting points are corrected.

(14) Microanalyses by Dr. S. M. Nagy and Associates, Massachusetts Institute of Technology.

(15) G. La Parola, *Gazz. chim. ital.*, **64**, 919 (1934).

acetate (2.46 g.) and 3.0 g. of maleic anhydride and glacial acetic acid (75 cc.) was stirred for two hours. The pink suspension turned yellow during the reaction. The reaction mixture was diluted with 800 cc. of ice-water while stirred. The precipitate was collected, air-dried and recrystallized from alcohol and water to give III as a hydrate in glistening golden-yellow needles, yield 6.0 g. (68%), m.p. 99°, sintered to a red oil. After it was dried in a desiccator over calcium chloride overnight, the yellow sample had the composition of a dihydrate.

Anal. Calcd. for $C_{14}H_{15}NO_2 + 2H_2O$ (293.27): C, 57.33; H, 5.16; N, 4.78. Found: C, 57.72; H, 5.35; N, 4.61.

The anhydrous form can be prepared by gradually drying the sample in a desiccator over phosphorus pentoxide, first at room temperature overnight, then at 65° for eight hours. Recrystallization of this sample repeatedly from ethyl acetate gave the anhydrous form (IIIa) as small ruby red crystals, m.p. 145–146°. The red form remains unchanged by cooling at −72° overnight but can be transformed readily to the yellow dihydrate by exposure to moisture in a closed jar at room temperature.

Anal. Calcd. for $C_{14}H_{11}NO_4$ (257.24): C, 65.36; H, 4.31; N, 5.45. Found: C, 64.90; H, 4.54; N, 5.15.

N-(5-Hydroxy-1-naphthyl)-maleamic Acid (VIII).—This compound was prepared by a similar procedure from purified 5-amino-1-naphthol hydrochloride, in 85% yield, m.p. 152–153°, as yellow prisms.

Anal. Calcd. for $C_{14}H_{11}NO_4$ (257.24): C, 65.36; H, 4.31; N, 5.45. Found: C, 65.54; H, 4.43; N, 5.23.

N-(4-Acetoxy-1-naphthyl)-maleimide (IV).—A mixture of 0.7 g. of N-(4-hydroxy-1-naphthyl)-maleamic acid (III), 5 cc. of acetic anhydride, 0.1 g. of sodium acetate was heated for ten minutes over a steam-bath with exclusion of moisture. The reaction mixture was poured into cold water with stirring. Sodium bicarbonate was added to facilitate the precipitation. The solids were collected and recrystallized from chloroform and 95% ethanol in fine yellow needles, 0.5 g., m.p. 175–176°. Further recrystallization from the same solvent raised the melting point to 178–179°.

Anal. Calcd. for $C_{16}H_{11}NO_4$ (281.26): C, 68.32; H, 3.94; N, 4.98. Found: C, 68.24; H, 4.05; N, 5.08.

This compound does not couple with tetrazotized diorthoanisidine in a suspension of sodium bicarbonate solution or in dilute acetic acid. Attempts to selectively hydrolyze the ester linkage by the following methods were unsuccessful; methanol and sodium, methanol and ammonia at 0°, acetone and 0.4 *N* sodium hydroxide, 10% sodium carbonate and chloroform.

N-(4-Hydroxy-1-naphthyl)-maleimide (II).—A well ground mixture of 5.0 g. of purified 4-amino-1-naphthol hydrochloride,[16] 2.3 g. of sodium bicarbonate and 2.5 g. of maleic anhydride was fused at 158–160° for one hour and 40 minutes in a open flask at which time practically no further gas evolution was observed. The brown residue was first extracted with three portions (70 cc. each) of hot water and the residue was dissolved in 80 cc. of hot 95% ethanol and then separated from any insoluble material by filtration. After cooling and diluting with water the brown precipitate was immediately collected and dried at 70° *in vacuo* to give 2.7 g. (45%) of crude N-(4-hydroxy-1-naphthyl)-maleimide, m.p. 198° dec. Attempts to recrystallize the product from acetone–water, ethyl acetate, dilute alcohol, alcohol and ether gave a gummy product. The analytical sample was purified by dissolving in warm glacial acetic acid and adding water until precipitation was completed. The product retained its original tan amorphous appearance, m.p. 202° dec. It gave a blue color when coupled with tetrazotized diorthoanisidine in sodium bicarbonate suspension but not in dilute acetic acid. Attempts to prepare and purify larger batches resulted in lower yields. Attempts to remove water of crystallization by heating at 110° in a pistol resulted in darkening and decomposition of the product.

Anal. Calcd. for $C_{14}H_9NO_3 + \frac{1}{2}H_2O$ (248.23): C, 67.83; H, 4.05; N, 5.66. Found: C, 68.17, 68.19; H, 4.16, 4.28; N, 5.27.

Acetylation of (II) using acetic anhydride and sodium acetate did not yield IV but a different compound probably represented in formula IX (m.p. 181–182°, as light tan small crystals). Mixed melting point of IX with IV was 168–172°. The infrared spectrum differed, although only slightly in the fingerprint region. Compound IX was less soluble in chloroform than IV.

Anal. Calcd. for $C_{16}H_{11}NO_4$ (281.26): N, 4.98. Found: N, 4.99.

N-(5-Hydroxy-1-naphthyl)-maleimide (XI).—This compound was prepared in a similar manner to that described for II from 5-amino-1-naphthol hydrochloride. The yield of crude product was 62%. Recrystallization from ethanol and water gave a tan powder, m.p. 235° dec. After drying the analytical sample again for 1.5 hours at 100° over fresh phosphorus pentoxide, it lost 1.36% of the original weight.

Anal. Calcd. for $C_{14}H_9NO_3 + \frac{1}{2}H_2O$ (248.23): C, 67.83; H, 4.05; N, 5.66. Found: C, 67.65; H, 4.39; N, 6.00.

N-(4-Hydroxy-1-naphthyl)-isomaleimide (V).—To an 8-g. sample of N-(4-hydroxy-1-naphthyl)-maleamic acid (III) in 50 cc. of dry dioxane was added 5 cc. of trifluoroacetic anhydride in the cold and allowed to stand for one hour at room temperature. The deep red solution was diluted with 200 cc. of ice-water and the brown precipitate was collected and washed well with cold water to give 6.6 g. of the crude isomaleimide, m.p. 171–173° dec. (yield 86%). Recrystallization twice from acetone and water afforded the pure product as a vermillion powder, m.p. 173–174° dec. after drying *in vacuo* at 100° over phosphorus pentoxide. This product gave a positive Baeyer test, ferric chloride test and coupled with tetrazotized diorthoanisidine in bicarbonate suspension but not in dilute acetic acid. When to an alcoholic solution of this compound was added a drop of 1 *N* sodium hydroxide, a wine red color appeared which changed to purple and then faded within minutes to a straw-yellow color.

Anal. Calcd. for $C_{14}H_9NO_3$ (239.22): C, 70.29; H, 3.79; N, 5.86. Found: C, 70.60; H, 3.98; N, 5.70.

When the same procedure was employed in an attempt to prepare the isomaleimide from N-(1-naphthyl)-maleamic acid (VII) and from N-(5-hydroxy-1-naphthyl)-maleamic acid (VIII), the starting compound was recovered unchanged.

Ultraviolet spectra were determined by a Cary automatic recording spectrophotometer, model 11M, Applied Physics Corp., Pasadena, Calif. The solvent used was 95% ethanol, reagent grade. The principal maxima and the corresponding extinction coefficients are summarized in Table I.

Infrared spectra were determined by a Perkin–Elmer model 21 double beam spectrophotometer. The main bands are summarized in Table I, together with any necessary assignments.

Histochemical Experiments.—The histochemical method consisted of treating the protein-bound sulfhydryl groups in sections of tissue with one or another of the reagents at *p*H 8.0. After extraction of the excess reagents, an azo dye was produced at the site of the reaction by coupling with tetrazotized diorthoanisidine. Sections of skin were primarily used as test objects since the keratinous proteins in the epidermis and hair are rich in sulfhydryl groups. In addition, the effects of sulfhydryl inhibitors were assayed on the histochemical reactions and in each instance the staining produced by each reagent was compared with the staining produced on parallel sections with 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD), a specific sulfhydryl reagent.[2,3]

The N-(4-hydroxy-1-naphthyl)-isomaleimide (V), 1,4-naphthoquinone monobenzenesulfonimide (VI) and N-(4-hydroxy-1-naphthyl)-maleimide (II) gave an intense reaction identical with the earlier reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide. The reactions of all four of these compounds were inhibited completely if the tissue sections were treated first with iodine, iodoacetate, iodoacetamide or N-ethylmaleimide. The reactions with the four compounds was reversed by treatment of the sections with an excess of glutathione in solution before coupling with tetrazotized diorthoanisidine. No histochemical reaction was produced with any of the maleamic acid compounds.

N-(1-Naphthyl)-maleimide (X) or N-(4-acetoxy-1-naphthyl)-maleimide (IV) also reacted with sulfhydryl groups as demonstrated by their ability to block the reaction with the chromogenic reagents. However, these two compounds could be used also as histochemical reagents to stain protein-

(16) L. F. Fieser, "Organic Syntheses," Coll. Vol. II, John Wiley & Sons, Inc., New York, N. Y., 1943, p. 39.

TABLE I

MALEIMIDE DERIVATIVES

	Ultraviolet spectra λ_{max}, mμ	$\epsilon \times 10^{-3}$	Infrared spectra,[a] μ
N-Ethylmaleimide (I)	217	12.74	λchf: 5.84(s), 6.27(w), 6.92(s), 7.12, 7.22, 7.40, 7.48(w), 9.04, 9.20(w), 10.28(s), 10.57
	224	10.75	
	310	0.51	
N-(1-Naphthyl)-maleimide (X)	223	104.70	λchf: 5.73(sh), 5.80(s), 6.24(m), 6.83(vw), 7.10(s), 7.28(s)
	270	11.02	
N-(4-Acetoxy-1-naphthyl)-maleimide (IV)	223	58.14	λchf: 5.65(vw), 5.80(s), 6.24, 6.82, 7.11, 7.50, 8.71, 9.24 (vw)
	286	7.48	
N-(4-Hydroxy-1-naphthyl)-maleimide (II)	212	32.40	λDioxane: 5.70(vw), 5.86, 6.12(vw), 6.27(s)
	232	23.82	
	298	5.61	
	323	3.43	
N-(5-Hydroxy-1-naphthyl)-maleimide (XI)	210	32.90	λDioxane: 3.15(s), 5.68(w. sh), 5.86(s), 6.25(s), 6.58
	234	30.10	
	314	7.05	
	326	6.58	
N-(4-Hydroxy-1-naphthyl)-isomaleimide (V)[b]	209	38.90	λDioxane: 3.10, 5.57(s), 5.99, 6.28(s), 6.40(w)
	237	27.90	
	280	8.52	
	310	5.12	
Naphthoquinone monobenzenesulfonimide (VI)	236	20.20	λchf: 5.96(s), 6.15(s), 6.24(w), 6.44–6.46(b), 6.90(w)
	256	9.72	
	305	12.43	
N-(1-Naphthyl)-maleamic acid (VII)	222	52.30	λDioxane: 3.08–3.80(b), 5.76(vw), 5.90(w), 6.10(s), 6.48(s).
	292	5.42	λKI: 3.10, 3.30, 5.86, 6.12–6.70(b), 7.20(s), 7.44, 7.68,
	313	4.96	7.88, 8.28, 9.86(w), 10.32(s), 11.84(s), 12.76, 13.16
N-(4-Hydroxy-1-naphthyl)-maleamic acid (anhydrous, red) IIIa	209	55.50	λKI: 3.10(b), 5.90(s), 6.20–7.0(b), 7.20(w), 7.40(w), 9.90,
	237	31.06	10.30, 11.10(s), 11.68(s), 11.82(s), 12.10, 12.20(s), 12.70
	325	8.20	(s)
Dihydrate, yellow (IIIb)	209	37.10	
	237	22.70	
	327	5.56	
N-(5-Hydroxy-1-naphthyl)-maleamic acid (VIII)	222	47.00	λKI: 3.10–3.20(b), 5.95(s), 6.14–6.94(b), 7.46, 8.0, 9.35
	316	6.65	10.84(s), 11.16, 11.80, 12.85, 13.6
	328	6.92	

[a] The notations for the ultraviolet data are braces = shoulder and for the infrared data are s = strong, w = weak, b = broad, sh = shoulder. The medium bands are not indicated. [b] When 0.1 *N* sodium hydroxide was added, the maxima shift to 210, 250, 336 mμ with relatively low intensity. Owing to the nature of the intermediate as discussed in the text, no quantitative measurement was possible.

bound sulfhydryl groups. The acetoxy derivative, after reaction with protein-bound sulfhydryl groups, was hydrolyzed by brief exposure to strong alkali and subsequent coupling to an azo dye resulted in demonstration of sulfhydryl groups. Even N-(1-naphthyl)-maleimide, after reaction with protein-bound sulfhydryl groups in tissue, coupled with tetrazotized diorthoanisidine to produce a bright yellow azo dye. Coupling occurred presumably on the maleimide ring. Both of these reactions could be blocked by N-ethylmaleimide, iodine or iodoacetate.

Acknowledgment is due to Miss Margaret Pei-Chuan Lee and Miss Nancy MacAllaster for technical assistance. R. J. Barrnett collaborated on the histochemical studies.

CAMBRIDGE 38, MASS.,
BALTIMORE 5, MD.