solution of the product obtained in a preparative bromination of *tert*-butylbenzene in trifluoroacetic acid. Known mixtures (similar in composition to the product mixtures) of the isomeric bromo-*tert*-butylbenzenes were used to define the vpc response factors. Replicate analyses were obtained on each reaction product. The results are presented in Table I.

TABLE I

ISOMER DISTRIBUTIONS IN THE BROMINATION OF *tert*-BUTYLBENZENE AT 25°

Solvent,[a] %	Concentration, M: C_6H_5-C_4H_9]	[Na-Br]	[Br_2]	Isomer distribution, mol %: Ortho	Meta	Para
			Trifluoroacetic Acid			
100	0.10	0.09	0.03	0.00	0.35 ± 0.10	99.65 ± 0.10
100	0.10	0.09	0.03	0.00	0.36 ± 0.10	99.64 ± 0.10
100				Av 0.00	0.35 ± 0.01	99.65 ± 0.01
93.3	0.10	0.09	0.03	0.00	0.43 ± 0.10	99.57 ± 0.10
93.3	0.10	0.09	0.03	0.00	0.37 ± 0.10	99.63 ± 0.10
93.3	0.10	0.09	0.09	0.00	0.51 ± 0.10	99.49 ± 0.10
93.3				Av 0.00	0.44 ± 0.07	99.56 ± 0.07
78.3	0.10	0.09	0.03	0.00	0.34 ± 0.10	99.66 ± 0.10
78.3	0.05	0.045	0.015	0.00	0.34 ± 0.10	99.66 ± 0.10
78.3				Av 0.00	0.34 ± 0.00	99.66 ± 0.00
			Acetic Acid			
85	0.51	0	0.13	1.26 ± 0.20	1.72 ± 0.20	97.02 ± 0.20
85[b]	0.51	0	0.13	1.20	1.47	97.3

[a] Weight per cent of acid. [b] Analysis by infrared spectroscopy: H. C. Brown and L. M. Stock, *J. Amer. Chem. Soc.*, **81**, 5615 (1959).

The partial rate factors for the bromination reaction are presented in Table II.

TABLE II

Solvent[a]	Partial rate factor: Toluene[b] o_f^{Me}	m_f^{Me}	p_f^{Me}	*tert*-Butylbenzene[c] $m_f^{t\text{-Bu}}$	$m_f^{t\text{-Bu}}$	$p_f^{t\text{-Bu}}$
			Trifluoroacetic Acid			
100	1360	10[d]	12,700		34	19,200
93.3	4340		42,400		119	59,100
78.3[e]	2150		19,300		35	20,000
			Acetic Acid			
85.0	600	5.5	2,420	5.2	7.3	805

[a] Weight per cent of acid. [b] Factors for trifluoroacetic acid, ref 3 and 4. Factors for acetic acid: H. C. Brown and L. M. Stock, *J. Amer. Chem. Soc.*, **79**, 1421 (1957). [c] Factors for trifluoroacetic acid are based on the rate data of ref 3 and 4 and the isomer distributions shown in Table I. Factors for acetic acid are based on the rate data of ref 7 and the isomer distributions shown in Table I. [d] Determined by additivity method, ref 3. [e] Note ref 9.

The isomer distributions measured in this study establish that $m_f^{t\text{-Bu}}$ is very large for the bromination reaction in the three solvents rich in trifluoroacetic acid. Indeed, $m_f^{t\text{-Bu}}$ for 93.3% acid is the largest value thus far obtained.[9] These results suggest, as discussed previously[5] for the tritium exchange and chlorination reaction, that the reversal in the relative reactivity at the para position of toluene and *tert*-butylbenzene is, in significant part, the consequence of the selective increase in the free energy of *tert*-butylbenzene in the trifluoroacetic acid solvents.

(9) It is pertinent that there is an uncertainty in the rate constant for the bromination of benzene in 78.3% trifluoroacetic acid.[4] However, there is no uncertainty in the rate data for the bromination of the alkylbenzenes in 93.3% acid.[4]

Experimental Section

tert-Butylbenzene (Phillips, research grade) was used without further purification. Trifluoroacetic acid (Matheson Co.) was used with and without fractionation. There were no discernible differences in the isomer distributions. The bromo-*tert*-butylbenzenes were prepared *via* the *tert*-butylation of acetanilide and subsequent deamination.[10] Highly purified samples were employed to standardize the analytical method. The reaction conditions adopted for the prior work[3,4] were used in this study. The products were isolated in the usual way and analyzed most effectively on either Apiezon L or Carbowax 20M columns (50 m) operated at 160° with a 0.5 ml min^{-1} He flow using a Varian Series 1200 chromatograph equipped with a flame ionization detector.

Registry No.—*tert*-Butylbenzene, 98-06-6.

(10) T. F. Crimmins, Thesis, Purdue University Library, 1966.

The Alkaline Decomposition of Organic Disulfides. IV. A Limitation on the Use of Ellman's Reagent, 2,2′-Dinitro-5,5′-dithiodibenzoic Acid

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About ten years ago Ellman[3] described an ingenious procedure for determining quantitatively sulfhydryl content. An excess of the reagent, 2,2′-dinitro-5,5′-dithiodibenzoic acid sodium salt, reacts by thiol–disulfide exchange to release a 2-nitro-5-mercaptobenzoate anion for each sulfhydryl group present. While the disulfide reagent has only a pale yellow color, the 2-nitro-5-mercaptobenzoate anion, like all nitrothiophenolate anions, has a deep color so that measurement of absorbance at 412 nm, as specified by Ellman, when referred to a standard, is a quantitative measure of the sulfhydryl groups originally present.

Ellman's reagent, specifically, is a 10^{-2} M solution of the disulfide in phosphate buffer (μ = 0.1) at pH 7.0. The sample to be analyzed is mixed with phosphate buffer at pH 8.0 before addition of the reagent. The reasons for the choices of pH, though not explicitly stated, are two: the dithiodicarboxylic acid is scarcely soluble in water though its sodium salt is readily so, and the mercaptide ion is much more highly colored than its conjugate acid.

Since the determination of sulfhydryl groups is a frequently employed procedure and Ellman's method is a very attractive one, it has been cited hundreds of times during the last decade. Thus, it is worthwhile to call attention to a hitherto almost unmentioned fact, the extreme sensitivity of Ellman's reagent to alkali, which could lead to erroneous results. Donovan[4] has noted that "Ellman's reagent... showed absorption changes in alkali [concentration not specified] very

(1) Postdoctoral Research Associate, 1969–1970.

(2) Participant in the National Science Foundation Undergraduate Research Participation Program, 1969.

(3) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

(4) J. W. Donovan, *Biochem. Biophys. Res. Commun.*, **29**, 734 (1967).

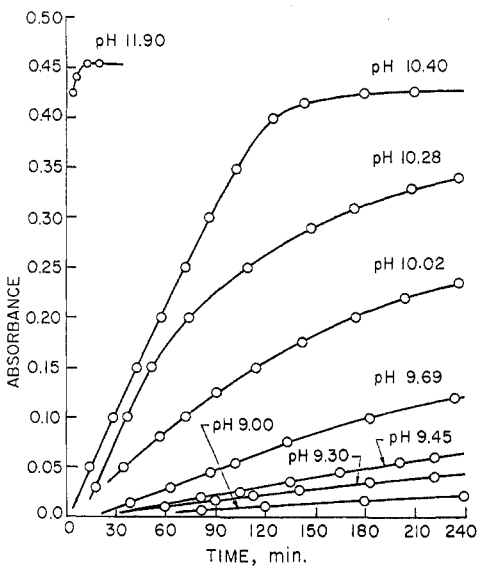
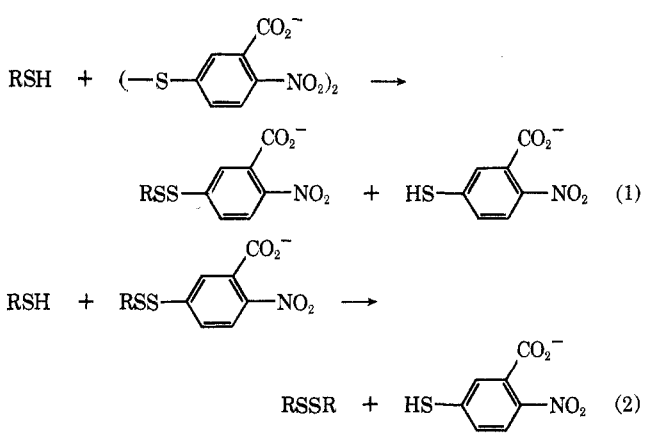


Figure 1.—In each case 0.1 ml of Ellman's reagent (10^{-2} M in 10^{-1} M phosphate at pH 7.02) was brought to 50 ml with 0.25 M phosphate solution at the pH value shown.

similar to those observed upon reduction. The absorption change at 412 mμ was 0.73 of that observed upon reduction, suggestive of S–S fission." It should be emphasized that the reliability of the method, when used in accord with Ellman's protocol, is not questioned. Benedict and Stedman,[5] however, have noted explicitly that a number of other nucleophiles interfere in the determination of thiol groups with Ellman's reagent: cyanide, sulfite, hydrosulfide, thiosulfate, and dithionite.

Recently, Danehy and Parameswaran[6] reported an inverse correlation between the relative sensitivity of organic disulfides to alkaline decomposition and the pK_a values of the thiols which are acids conjugate to the thiolate anions displaced by the nucleophilic attack of the hydroxide ion. The more sensitive the disulfide is to alkali, the more acidic the corresponding thiol. 4-Nitrophenyl disulfide, of which Ellman's disulfide is a derivative, was the most sensitive one examined.

While 1 mol of Ellman's disulfide, by thiol–disulfide exchange, gives 1 mol of 2-nitro-5-mercaptobenzoate, or, in the presence of excess thiol, 2 mol of the absorbing species, the action of hydroxide ion on 2 mol of the disulfide should give 3 mol of the thiol, according to the stoichiometry already established for this kind of reaction.[6]



$$2RSSR + 4OH^- \longrightarrow 3RS^- + RSO_2^- + 2H_2O \quad (3)$$

The development of absorbance at 412 nm in aqueous solutions of 2,2′-dinitro-5,5′-dithiodibenzoate at pH values established over the range of 9–12 has now been followed at room temperature. From the results (Figure 1) it can be seen, as might have been expected from the earlier report,[6] that near pH 12 alkaline decomposition is complete within 15 min. At pH 9.30 decomposition is about 9% in 4 hr. Even as low as pH 8.00, at which sulfhydryl determinations are made, not shown on the graph, about 5% decomposition takes place within 48 hr. Ellman's reagent itself (10^{-2} M disulfide, pH 7.0) develops no absorbance at 412 nm in 7 weeks and may be stable for much longer periods of time.

Grassetti and Murray[7] have reported that "At pH 3.3 no reaction occurred between DTNB [2,2′-dinitro-5,5′-dithiodibenzoic acid] and cysteine; however, when the pH of the medium was increased, theoretical SH values were obtained in the range of pH between 7.8 and 10.4 . . . Absorbance (412 nm) was measured against a blank without cysteine." The time allowed for reaction was not reported by these workers. From Figure 1 it can be seen that at pH 10.4 about 11% of the disulfide has decomposed within 10 min.

In a prior publication Ellman[8] had reported that the p-nitrothiophenolate anion has an a_m = 13,600 at the λ_{max} 412 nm. In his definitive paper[8] (p 72), he assumes both of these values for the 2-nitro-5-mercaptobenzoate anion. Curiously, despite the extensive references to the corresponding disulfide, 2-nitro-5-mercaptobenzoic acid has never been reported.

It seemed to us worthwhile to prepare an authentic sample of 2-nitro-5-mercaptobenzoic acid and to determine its physical constants, especially the value for the molar absorptivity (a_m) of the thiolate anion at 412 nm. Samples of 2-nitro-5-mercaptobenzoic acid have been prepared by the action of aqueous alkali (reaction 3) or of aqueous sodium thioglycolate (reactions 1 and 2) on the disulfide, followed by precipitation and recovery. Elemental analyses (Table I) and

TABLE I
ELEMENTAL ANALYSES

Compd	C, % Calcd	C, % Found	H, % Calcd	H, % Found	N, % Calcd	N, % Found	S, % Calcd	S, % Found
2-Nitro-5-mercaptobenzoic acid	42.41		2.53		7.03		16.09	
A[a]		42.38		2.50		7.08		15.46
B[b]		42.13		2.43		6.66		18.06

[a] Product of the action of aqueous alkali on 2,2′-dinitro-5,5′-dithiodibenzoate. [b] Product of the action of aqueous thioglycolate on 2,2′-dinitro-5,5′-dithiodibenzoate.

absorption spectra indicate that they are essentially the same compound. Melting point ranges, low iodine titers, and the fact that the absorption spectra of aqueous solutions unprotected from the air change

(5) R. C. Benedict and R. L. Stedman, *Analyst (London)*, **95**, 296 (1970).
(6) J. P. Danehy and K. N. Parameswaran, *J. Org. Chem.*, **32**, 568 (1968).
(7) D. R. Grassetti and J. F. Murray, Jr., *Arch. Biochem. Biophys.*, **119**, 41 (1967).
(8) G. L. Ellman, *ibid.*, **74**, 443 (1958).

rapidly to resemble those characteristic of the disulfide, indicate that 2-nitro-5-mercaptobenzoic acid is exceedingly sensitive to aerial oxidation.

Since it was not practical to prepare a sample of pure 2-nitro-5-mercaptobenzoic acid, its molar absorptivity was calculated from the absorbance of a solution of the disulfide in aqueous phosphate buffer to which sufficient aqueous solution of sodium thioglycolate had been added to produce maximal absorbance (Table II). A solution of exactly the same

TABLE II

ABSORPTION SPECTRAL CONSTANTS FOR CERTAIN AROMATIC DISULFIDES AND THE CORRESPONDING THIOLS

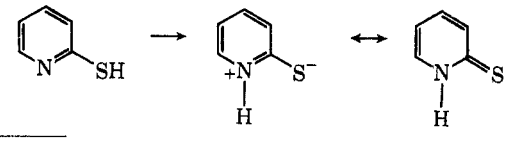
Compd	Registry no.	λ_{max}, nm	a_m
2,2′-Dinitro-5,5′-dithiodibenzoate[a]	552-24-9	325	17,500
		748	2,800
2-Nitro-5-mercaptobenzoate[b]	18430-02-9	412	13,600
		825	12,600
2,2′-Dithiodipyridine[c]	2127-03-9	233	13,900
		281	9,700
2-Mercaptopyridine[c]	2637-34-5	238	6,200
		343	8,700
		740	550
4,4′-Dithiodipyridine[d]	2645-22-9	247	16,100
4-Mercaptopyridine[d]	4556-23-4	230	9,600
		324	19,800
2,2′-Dithiodipyrimidine[c]	15718-46-4	237	19,000
2-Mercaptopyrimidine[c]	1450-85-7	278	21,000
		346	2,600
		780	795

[a] In aqueous phosphate buffer at pH 7.0. [b] The above disulfide solution to which sufficient sodium thioglycolate had been added to give maximal absorbance. [c] 0.1 N H_2SO_4. [d] Phosphate buffer at pH 7.2.

disulfide concentration, but 0.1 N in NaOH, gave exactly 0.75 of the absorbance of the previous solution, completely in agreement with reaction 3 and the observation of Donovan.[4]

The pK_a for the sulfhydryl group at 25°, determined spectrophotometrically, was found to be 4.75. Harrap[9] has reported a value of 4.8 ± 0.1 at 20°. From the recorded values[10] for 4-nitrothiophenol (4.77 at 30° in 40% aqueous ethanol) and for 3-mercaptobenzoic acid (6.15 at 28° in water) it is clear, as was expected, that the nitro group increases the acidity of thiophenol considerably more than does the carboxyl group.

An exactly parallel situation is presented by 2,2′- and 4,4′-dithiodipyridine and their nitro and carboxy derivatives, all of which have been recommended by Grassetti and Murray[7,11] as alternatives to Ellman's reagent for the determination of sulfhydryl. Albert and Barlin[12] have shown that 2- and 4-mercaptopyridine are uncommonly acidic thiols, by reason of the resonance



stabilization of the highly favored tautomer (pK_a values of −1.07 and +1.43, respectively). One would expect that the corresponding disulfides would be at least as susceptible to alkaline cleavage as Ellman's reagent, and such has proved to be the case (see Table III).

TABLE III

DECOMPOSITION OF SEVERAL HETEROCYCLIC DISULFIDES IN AQUEOUS SOLUTION AT 25° AS A FUNCTION OF pH

Compd	pH	Half-life, min
2,2′-Dithiodipyridine[a]	11.20	12
	10.60	58
	10.32	200
	9.92	>300
4,4′-Dithiodipyridine[b]	11.32	13
	10.52	67
	10.43	97
	9.83	>300
2,2′-Dithiodipyrimidine[c]	11.40	4
	10.40	40
	9.92	133
	9.55	>240

Decomposition followed by measurement of increase of absorbance at [a] 740 nm, [b] 324 nm, [c] 780 nm.

Experimental Section

Materials.—2,2′-Dinitro-5,5′-dithiodibenzoic acid was purchased both from Calbiochem, Los Angeles, Calif., and Aldrich Chemicals, Milwaukee, Wis. 2- and 4-Mercaptopyridine were purchased from Aldrich Chemical Co. 2-mercaptopyrimidine was obtained from Research Organic/Inorganic Chemicals, Sun City, Calif. Thioglycolic acid was a gift from Evans Chemetics, New York City. The disulfides were prepared by oxidizing aqueous solutions of the thiols with potassium triiodide: 2,2′-dithiodipyridine melted at 56–58° (lit. 57–58°); 4,4′-dithiodipyridine melted at 74–76° (lit. 74°); 2,2′-dithiodipyrimidine melted at 134–137° (lit. 139–140°).

Methods.—All melting points are uncorrected. Absorbance measurements given in Figure 1 were obtained with a Bausch & Lomb spectronic 20. Absorbance measurements required for determination of λ_{max} values, calculation of a_m values, and determination of pK_a values were obtained with a Beckman DB-G recording spectrophotometer.

Registry No.—2,2′-Dinitro-5,5′-dithiodibenzoic acid, 69-78-3.

Acknowledgment.—Grateful acknowledgment is made to National Institutes of Health (AM-13109) and to the National Science Foundation Undergraduate Research Participation Program.

(9) K. R. Harrap, *Biochem. Pharmacol.*, **16**, 725 (1967).

(10) J. P. Danehy and K. N. Parameswaran, *J. Chem. Eng. Data*, **12**, 386 (1968).

(11) D. R. Grassetti and J. F. Murray, Jr., *Anal. Biochem.*, **21**, 427 (1967); D. R. Grassetti, J. F. Murray, Jr., and H. T. Ruan, *Biochem. Pharmacol.*, **18**, 603 (1969); D. R. Grassetti and J. F. Murray, *J. Chromatogr.*, **41**, 121 (1969); D. R. Grassetti and J. F. Murray, *Anal. Chim. Acta*, **46**, 139 (1969); J. N. Mehrishi and D. R. Grassetti, *Nature*, **224**, 563 (1969).

(12) A. Albert and G. B. Barlin, *J. Chem. Soc.*, 2384 (1959).

Synthesis of β-Substituted Pyrroles *via* 1-(Pyrrol-2-ylmethylene)pyrrolidinium Salts

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We wished to prepare 3-isoprenoid pyrroles for screening as arthropod antimaturants.[1] Such materials would be pyrrolic analogs of perillen and dendrolasin, substances isolated from the mandibular glands of an

(1) C. M. Williams, International Symposium on New Perspectives on the Control of Injurious Insects, Rome, Italy, Sept 16–18, 1968.