which a part of this work was carried out. This investigation also was supported in part by a grant from the National Science Foundation.

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[CONTRIBUTION FROM DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

Studies on Thiols. I. Oxidation of Thiol Groups by 2,6-Dichlorophenol Indophenol[1]

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RECEIVED NOVEMBER 5, 1954

Using a spectrophotometric method, the oxidation of a variety of thiols by the dye, 2,6-dichlorophenol indophenol, has been investigated. Depending upon the structure of an individual thiol, the reaction rate is either relatively fast and approaches a final stoichiometry of oxidant : reductant reacting of approximately 1:1 or slow, whereupon the stoichiometry approaches approximately 1:2. The reaction rate is directly proportional to the concentration of both oxidant and reductant and hydrogen ion concentration. Metal ions, such as Cu^{++}, Ni^{++} and Fe^{++} depress the rate and shift the stoichiometry toward a 1:2 reaction; Versene reverses this effect. In light of these kinetic studies the mechanism of reaction is discussed, and a quantitative assay for purified thiols has been developed. Acyl thiols, disulfides and certain other sulfur-containing compounds do not react in this system. "Potential" thiols, such as thiazolidines, thiazolines and thiazoles, react with indophenol, but with a rate several orders of magnitude slower than "open" thiols.

The importance of thiol[3] groups in biological systems[4] is due to the widespread occurrence of this functional group in many proteins and in simple molecules such as glutathione, cysteine, Coenzyme A[5] and lipoic acid. In addition to the well-known reversible oxidation–reduction to the disulfide stage, thiol groups have recently taken on additional significance as carriers of acyl groups in enzymatic reactions.[6]

The chemistry of thiol groups, however, still contains areas of uncertainty, especially with regard to oxidation processes.[7] This difficulty arises in part from the fact that thiols can undergo oxidation to a series of higher states.[8] Oxidation of thiols is also affected by factors[4] such as: (a) steric and inductive effects of other groups in the molecule, which may shield the thiol group, cause hydrogen bonding or cause ring formation; (b) the presence of metal ions and (c) the nature of the oxidant.

The present investigation was undertaken to gain further information pertinent to the above problems. The dye, 2,6 dichlorophenol indophenol, was chosen as the oxidant, since it permits the reaction to be followed spectrophotometrically. Although the oxidation of thiols by this dye may be complicated by non-oxidative side reactions (*e.g.*, addition of the thiol to the quinoid form of the dye[4]), the rapidity of the oxidation–reduction reaction in dilute solutions minimizes this difficulty, and makes it possible to obtain data on the kinetics and stoichiometry of a typical oxidative reaction involving thiols.

Experimental

Chemicals.—All chemicals were purchased from commercial sources unless otherwise specified.

Pantethine was kindly supplied by Dr. Bird of Parke, Davis Co. and alatheine (β-alanylcysteine) from Drs. Cheldelin and King. Thioglycolic acid, a redistilled Merck product, and Versene from the Bersworth Chemical Co., were generously supplied by Dr. P. E. Wilcox.

Thiazolidine-4-carboxylic acid was prepared by the method of Ratner and Clarke[9] (m.p., with decomposition, 197–200°). L-Cystine disulfoxide was synthesized by the method of Toennies and Lavine[10] and converted to cysteinesulfinic acid by the method of Lavine[11]; the purity of both compounds was determined iodometrically. Perbenzoic acid (used in the preparation of cystine disulfoxide) was prepared by the method of Braun.[12] 2-Methyl-Δ^2-thiazoline was obtained from L. Light and Co., Ltd., or synthesized by the method of Gabriel and Hirsch.[13] Both products had physical constants (b.p. and m.p. of the picrate) slightly lower than those listed in Beilstein[14,15] and may have contained the 5-methyl isomer as an impurity.

Colorimetric Assay of Thiols.—The following system was used in all assays of thiols except where noted.

10^{-3} *M* Indophenol	0.15 ml.
10^{-1} *M* Phosphate buffer, *p*H 7	1.00 ml.
Water	1.85 ml.
1.25×10^{-2} *M* Thiol	0.01 ml.

The indophenol, buffer and water are mixed in a 1-cm. cuvette, and the log I_0/I value read in the Beckman spectrophotometer, model DU, at 600 mμ against a blank composed of buffer and water. The log I_0/I (or E_{600}) remains constant with time as long as no reducing substance is added. At zero time, 0.01 ml. of thiol is added to both experimental and blank cuvettes and the decrease in E_{600} followed at 15- or 30-seconds intervals. Initial velocity is defined

(1) This material is taken from the Dissertation of Robert E. Basford offered in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The work was supported in part by grants from Eli Lilly and Co. and by Initiative 171, State of Washington. A preliminary report of this investigation has been given at the 37th Meeting of the Federated Societies of American Biologists at Chicago in April 1953 (F. M. Huennekens and R. E. Basford, *Federation Proc.*, **12**, 221 (1953)).

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(3) The term "thiol" will be used in preference to "sulfhydryl groups" or "mercaptans."

(4) For excellent reviews of thiol compounds and their role in biological processes, see E. S. G. Barron, *Adv. Enzymol.*, 11, 201 (1951); C. Fromageot, *ibid.*, **7**, 369 (1947).

(5) Abbreviations used in this and the subsequent paper include: CoA, Coenzyme A; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; indophenol, 2,6-dichlorophenolindophenol; CSH, CSSC, reduced and oxidized cysteine; GSH, CSSG, reduced and oxidized glutathione; RSH, thiol.

(6) F. Lynen and E. Reichert, *Angew. Chem.*, **63**, 47 (1951).

(7) For example, Freedman and Corwin (*J. Biol. Chem.*, **181**, 601 (1949)) have reviewed recently the widely divergent values which have been reported in the literature for the oxidation potential of the cysteine–cystine couple.

(8) For cysteine (RSH), the higher oxidation states are cystine (RSSR), cysteinesulfenic acid (RSOH), cysteinesulfinic acid (RSO_2H) and cysteic acid (RSO_3H).

(9) S. Ratner and H. Clarke, THIS JOURNAL, **59**, 200 (1937).

(10) G. Toennies and T. F. Lavine, *J. Biol. Chem.*, **113**, 571 (1936).

(11) T. F. Lavine, *ibid.*, **113**, 583 (1936).

(12) G. Braun in "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941, p. 431.

(13) S. Gabriel and C. F. von Hirsch, *Ber.*, **29**, 2609 (1896).

(14) "Beilstein," Fourth Ed., Vol. 27, p. 13 (1937).

(15) Ref. 14, Fourth Ed. Suppl., p. 206 (1938).

as μ moles of indophenol reduced/ml./min. and is calculated from the change in E_{600} over the first half minute. The total amount of thiol oxidized is assumed to be equal on a molal basis to the total amount of dye reduced and is obtained by subtracting the final E_{600} from the E_{600} at zero time and dividing by the molar extinction coefficient, E, for indophenol. Percentage oxidation is the ratio of the amount of dye reduced to the amount of thiol added, multiplied by 100.

Typical results using 0.125 μM of cysteine and glutathione are shown in Table I. Reoxidation of leuco indophenol at the end of the reaction (at pH 7) is negligible, since the values level off and remain at a constant value for long periods of time unless there is contamination by metal ions in the assay system (*cf.* Fig. 3).

TABLE I

RESULTS OF AN INDOPHENOL ASSAY

Assay method as described in the Experimental section.

Time, min.	Cysteine E_{600}	Glutathione	Time, min.	Cysteine E_{600}	Glutathione
0	0.980	0.990	5		0.488
0.5	.535	.750	6		.482
1	.422	.640	7		.482
2	.412	.550	8		.490
3	.421	.515	10	0.412	.500
4		.497			
			V_0, μmole/ml./min.	.047	.025
			Thiol oxidized, μmole	.089	.080

For compounds which required the exclusion of oxygen, the protocol was the same as above, except that the amount of all ingredients was doubled and the reaction was carried out in a small flask which could be evacuated and the solution then tipped into a cuvette connected by Tygon tubing.[16]

Determination of the Molar Extinction Coefficient of Indophenol.—By measuring the absorbance of a solution of known concentration, the molar extinction coefficient at 600 mμ of monosodium 2,6-dichlorophenol indophenol (Eastman Kodak Co.) at pH 7 was determined to be 1.91×10^7 cm.^{2}/mole. Several other commercial preparations of this dye gave lower E values and erratic results when used in the colorimetric assay.

Results and Discussion

General Characteristics of the Thiol–Indophenol Reaction.—A number of thiols were assayed with indophenol using the method described in the Experimental section. Table II shows the initial velocity and percentage oxidation of the compounds tested. Based upon their behavior in this simple assay system, the thiols could be grouped into two categories: (a) those which reacted rapidly and which, upon completion of the reaction, had bleached *nearly* an equimolar amount of dye,[17] and (b) those which reacted more slowly and ceased when *slightly more* than one-half the equimolar amount of dye had been bleached. Based upon the ratio of dye reduced to amount of thiol added, these reaction types will be referred to as "1:1 reaction" and "1:2 reaction." Examples of the two extreme cases are illustrated in Fig. 1 by thiosalicylic acid and thiophenol, respectively. The other compounds listed in Table II are intermediate in their behavior.

TABLE II

OXIDATION OF VARIOUS THIOLS BY INDOPHENOL

Assay system, V_0 and per cent. thiol oxidized are defined in the Experimental section.

Thiol	V_0, μmole/ml./min. $\times 10^2$	Thiol oxidized, %
Thiosalicylic acid	6.8	99
Thioglycolic acid	6.0	76
Thiophenol	5.4	65
Cysteine	2.9	82
Alatheine	3.1	86
Glutathione	2.7	67
β-Mercaptoethylamine	2.6	57
β-Mercaptoethanol	2.0	90
Thioacetic acid	1.6	67
Thiomalic acid	0.3	55
2,3-Dimercaptopropanol	5.7	109[a]

[a] On the basis of one –SH group oxidized, 54% on the basis of two –SH groups being oxidized.

(16) Designed and kindly furnished by Dr. W. B. Dandliker.

The fact that many of the thiols reacted almost stoichiometrically on an equimolar basis with indophenol was of interest, since if the thiol (RSH) were being oxidized to the disulfide (RSSR), the expected reaction would be

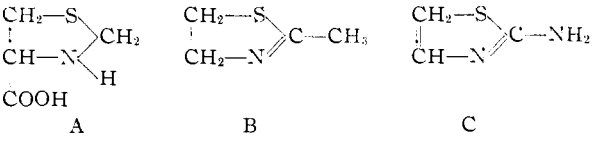
$$2RSH + I \longrightarrow RSSR + 1H_2 \qquad (1)$$

i.e., 2 molecules of thiol reacting with 1 molecule of dye. Todrick and Walker[18] in using indophenol to assay thiols also have reported that cysteine reacts with indophenol in a ratio of 1:1 but made no additional comment on the oxidation level of the product. As will be discussed subsequently in this paper, a 1:1 reaction would lead to a product at the oxidation level of a sulfenic acid.

Compounds which were not oxidized at all by indophenol include cystine, oxidized glutathione, S-acetylglutathione, pantathine, cysteic acid, cysteinesulfinic acid, benzenesulfinic acid, thioacetamide and biotin.

All of the reactions above were carried out in the presence of oxygen, which is a valid procedure, since the autoöxidation of sulfhydryl groups and of leuco indophenol is slow at pH 7, compared to the reaction rates being studied.

Potential Thiols.—In Table III are listed the reaction velocities of three "potential" thiols: thiazolidinecarboxylic acid (A), 2-methyl-Δ^2-thiazoline (B), and 2-aminothiazole (C), which have been shown to undergo hydrolysis with the liberation of a thiol group (*cf.* Ratner and Clarke[9] for A, Linderström-Lang and Jacobsen[19] for B, and Waters[20] for C).



A B C

Glutathione is included also in the table for comparison. It can be seen that the rate of oxidation of these three cyclic compounds is several orders of magnitude slower than that of free –SH compounds.[21] In the case of "potential thiols" the re-

(17) O. A. Bessey and C. G. King (*J. Biol. Chem.*, **103**, 687 (1933)) have shown that 2,6-dichlorophenol indophenol reacts stoichiometrically on a 1:1 basis with ascorbic acid and is, therefore, a 2-electron oxidant.

(18) A. Todrick and E. Walker, *Biochem. J.*, **31**, 292 (1937).

(19) K. Linderström-Lang and C. F. Jacobsen, *J. Biol. Chem.*, **137**, 443 (1941).

(20) W. A. Waters, "The Chemistry of Free Radicals," Clarendon Press, 2nd Edition, Oxford, 1948, p. 265.

(21) Both preparations of methylthiazoline, referred to in the Experimental Section, were oxidized at exactly the same rate by indophenol.

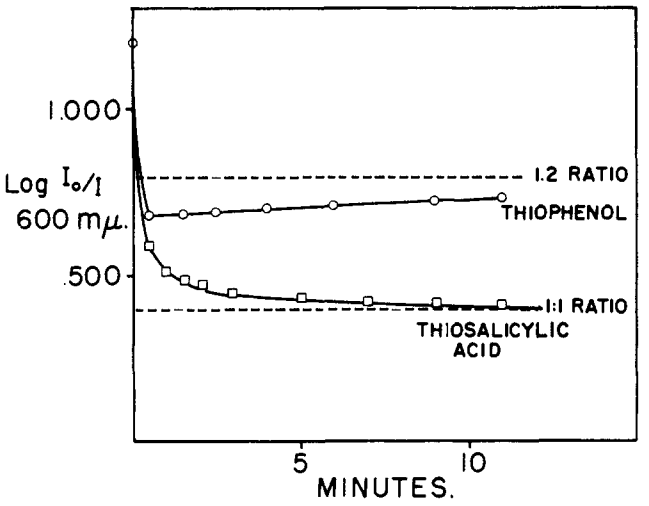


Fig. 1.—1:1 and 1:2 type reactions. Assays as described in the Experimental section, except that a slightly higher concentration of indophenol was used.

actions must be carried out in the absence of oxygen in order to avoid autoöxidation of thiols or leuco dye.

TABLE III

REACTIVITY OF "POTENTIAL" THIOLS WITH INDOPHENOL

Compound	V_0 (μmole/ml./min.)
Glutathione	2.7×10^{-2}
Thiazolidinecarboxylic acid[a]	2.1×10^{-4}
2-Methylthiazolidine[a]	2.8×10^{-5}
2-Aminothiazole[a]	2.4×10^{-6}

[a] Assayed in the absence of air as described in the Experimental section.

Kinetics of the Reaction.—A kinetic study of the indophenol reaction was made in order to obtain information regarding the mechanism. For this purpose, cysteine was chosen as a representative example of a "free" thiol which reacted in a *nearly* 1:1 fashion with the dye. Within the rather narrow range imposed by the spectrophotometric assay, the effect of indophenol concentration upon both the rate and final stoichiometry was investigated, and these data are shown in Table IV. For a given amount of thiol, 0.125 μmole, the initial velocity increases with indophenol concentration until a limiting value, imposed by the amount of reductant present, is reached. Conversely, at constant indophenol concentration, the variation in rate and stoichiometry with respect to cysteine concentration is given in Table V. In this case proportionality was again observed between initial velocity and amount of thiol added. No limiting value of initial velocity was reached, since the amount of indophenol present was always greater than the amount of thiol, in order to allow for complete reaction.

TABLE IV

EFFECT OF OXIDANT CONCENTRATION

Assay as described in the Experimental section. 0.125 μmole of cysteine added as reductant in all cases.

Indophenol, μmole × 10^2	$V_0 \times 10^2$, μmole/ml./min.	Δ Indophenol,[a] μmole × 10^2
5.0	1.2	5.0
9.1	2.4	8.3
15.5	3.2	11.0
18.8	3.6	12.4
23.4	3.7	12.7

[a] Δ Indophenol refers to the total amount of indophenol reduced at the completion of the reaction.

TABLE V

EFFECT OF REDUCTANT CONCENTRATION

Assay as described in the Experimental section. 0.150 μmole of dye added in all cases.

Cysteine, μmole × 10^2	$V_0 \times 10^2$, μmole/ml./min.	Δ Indophenol, μmole × 10^2
2.5	1.2	2.3
5.0	2.6	4.5
7.5	3.2	6.4
10.0	4.9	8.7
12.5	6.2	11.3

The effect of *p*H on initial velocity for cysteine, glutathione and CoA is shown in Fig. 2, where it is seen that in all cases the rate increases linearly as the *p*H is lowered. The fact that the reaction is directly proportional to the log of the H^+ concentration indicates that the reaction form of the thiol is RSH and not RS^-. It is not readily apparent why the slope of the cysteine curve is considerably greater than the slope of the curves for GSH and CoA. The effect is probably not due to the difference in the *pK* of the sulfhydryl group, since the *pK* of the –SH group in cysteine is 8.3 and in glutathione is 8.7.[22]

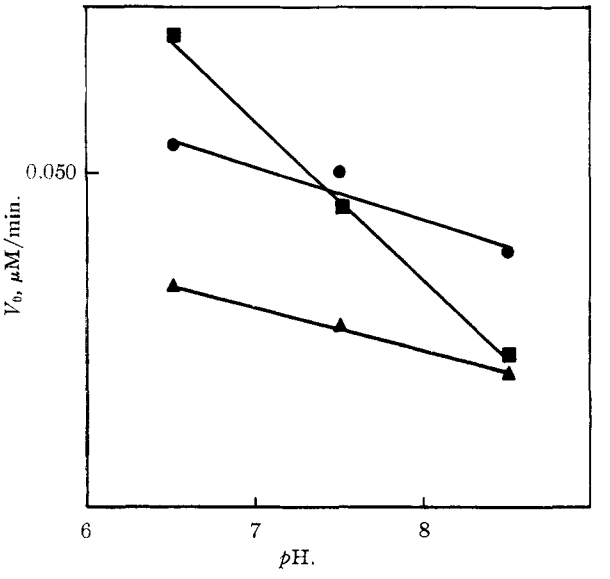


Fig. 2.—Effect of *p*H on the indophenol reaction. Assay as described in the Experimental section, except that phosphate buffers at the indicated *p*H values were used instead of *p*H 7: ▲, CoA SH; ●, GSH; ■, CSH.

Effect of Chelating Agent and Metal Ions.—Metal ions are known to affect markedly the autoöxidation of thiols, but in a seemingly inconsistent manner, since the same metal may act as a catalyst for the oxidation of certain thiols, and may have no effect on other thiols.[4]

(22) M. Calvin, in "Glutathione, A Symposium," Academic Press, Inc., New York, N. Y., 1954, p. 3. We are indebted to Professor Calvin for a copy of his manuscript prior to publication.

It was decided, therefore, to investigate the effects of both metal ions and a chelating agent, Versene, on the cysteine–indophenol reaction. Even though no metal ions have been added deliberately to the reaction mixtures, it is probable that traces are present in the reactants, buffer, or water, since the addition of Versene (10^{-3} M) to the system usually produced an increased velocity and an increase in the total amount of thiol oxidized (*cf.* Fig. 3). The presence of higher (1×10^{-2} M) concentrations of Versene is inhibitory.

On the basis of these results, it would be expected that the addition of metal ions to the system would produce an effect opposite to that of Versene. Figure 3 shows that equivalent concentrations of Cu^{++}, Fe^{++} and Mn^{++} depress both the rate and extent of the reaction. Also, in the presence of these metal ions, the autoöxidation of leuco indophenol is accelerated, causing the rate curve to turn upward. Since under the usual conditions the rate curves do not turn upward, but remain constant after the reaction is complete, the metals present, if any, as contaminants in the reagents are probably without effect.

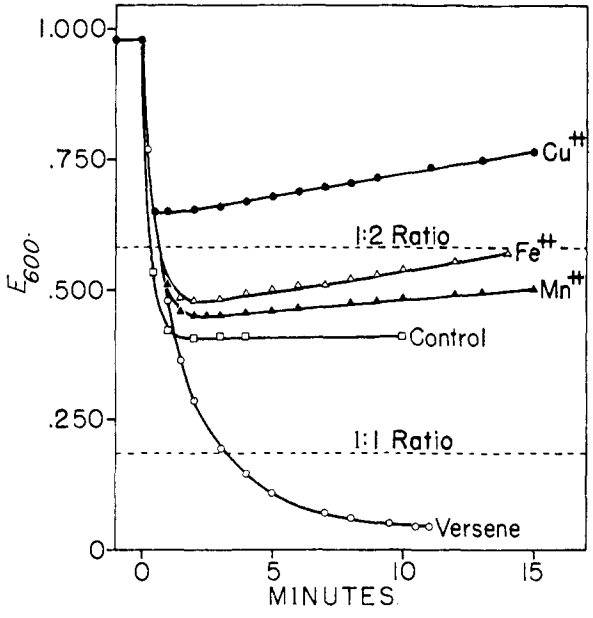


Fig. 3.—Effect of metal ions and Versene. Assay as described in the Experimental section, with metal ions present at a final concentration of 3.3×10^{-6} M and Versene at 10^{-3} M.

It is of interest that the metal ions displace the reactions toward a 1:2 ratio of dye to thiol, which is consistent with the proposed mechanism to be presented in the following section.

Stoichiometry and Mechanism of Reaction.—From the data presented above, it is seen that the oxidation of thiols by indophenol exhibits the following characteristics: (a) when the reaction velocity is rapid, the ratio of thiol added to indophenol reduced will approach 1, whereas when the rate is slow, the final ratio will approach 2; (b) the reaction rate is directly proportional both to the oxidant and reductant concentrations, and to the hydrogen ion concentration. These factors may be accounted for if it is assumed that the following mechanism holds for the reaction

$$RSH \longrightarrow RS^+ + 2e^- + H^+ \quad (2)$$

$$I + 2e^- + 2H^+ \longrightarrow IH_2 \quad (3)$$

Sum (1) and (2) $RSH + I + H^+ \longrightarrow RS^+ + IH_2$ (4)

where RSH and RS^+ are the thiol and its oxidation product at the level of a sulfenic acid, and I and IH_2 represent oxidized and reduced indophenol. If the thiol is of such a structure that electrons are readily abstracted from it, or that the oxidation product, RS^+, is stabilized by resonance, the reaction will proceed according to equation 4 fulfilling requirements (a) and (b) above. Furthermore, the non-reactivity of disulfides toward indophenol would indicate that the RS^+ stage may be achieved only by a two-electron loss from RSH, and not by a one-electron loss from RSSR.

Conversely, if the thiol is of such a nature that reaction (2) is slow, then it may be followed by

$$RS^- + RSH \longrightarrow RSSR + H^- \quad (5)$$

so that the over-all reaction will be represented by the sum of (2), (3) and (5), *viz.*

$$2RSH + I \longrightarrow RSSR + IH_2 \quad (1)$$

leading to the formation of the disulfide, RSSR.

If the observed effect of metal ions is assumed to be that of catalyzing reaction (5), the diminution of rate and displacement toward a 1:2 reaction is explained.

The oxidation product in this reaction, written for convenience as RS^+, may be either cysteinesulfenic acid or cystine monoxide (the dimer formed from the sulfenic acid by the loss of one molecule of water). These compounds have often been suggested as intermediates in the biological[23,24] or chemical[25–27] oxidation of cysteine, but owing to their extreme lability have never been isolated. From large-scale mixtures of cysteine and indophenol we have partially purified the reaction product. When assayed by means of paper chromatography in several solvent systems, the material is not identical with cysteine, cystine, cystinesulfinic acid or cysteic acid. However, the unequivocal identification of this material must await its isolation in pure form, and further characterization of its properties.

The indophenol–thiol reaction may be complicated further by a side reaction involving the interaction of the thiol with the indophenol molecule, since thiols have been shown to form addition products with quinones.[28] However, from the stoichiometry observed, the reaction probably does not occur appreciably in the spectrophotometric assays where the reactants are in a dilute solution and the expected oxidation–reduction reaction is almost completed within the first minute.

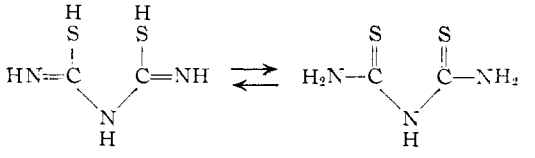
A first order plot of the cysteine–indophenol reaction yields a straight line for the first 60 seconds of

(23) G. Medes and N. Floyd, *Biochem. J.*, **36**, 259 (1942).
(24) N. W. Pirie, *ibid.*, **28**, 305 (1934).
(25) N. W. Pirie, *ibid.*, **27**, 1181 (1933).
(26) G. Toennies, *J. Biol. Chem.*, **120**, 297 (1937).
(27) G. Toennies, *ibid.*, **122**, 27 (1937).
(28) J. Troeger and A. Eggert, *J. prakt. Chem.*, **53**, 482 (1896); see also T. Posner, *Ann.*, **336**, 85 (1904).

the reaction. A plot of concentration against time also gives a straight line for the same period of time for all of the thiols tested. It is therefore valid to compare initial velocities of various thiols based on the first 30 seconds of the reaction instead of calculating rate constants in each case.

The finding that 2,3-dimercaptopropanol reacts rather rapidly, but with a final stoichiometry approaching 1:2 (*cf.* Table II) is difficult to explain by the above mechanism. While the juxtaposition of 2 thiol groups in the molecule would greatly enhance the probability of reaction (5) occurring as soon as the first thiol group of the molecule became oxidized to the sulfenic acid level, the resultant 4-membered ring would be unstable so that dimerization would be more probable. Lipoic acid (or 6,8-dithioöctanoic acid) on the other hand, in its reduced form, probably would be oxidized to the cyclic disulfide form by indophenol. Ferricyanide or various oxidation–reduction dyes have been used routinely as the terminal electron acceptor[29] in the assay of pyruvic oxidase which contains bound lipoic acid. The postulated mechanism[30] of the oxidative decarboxylation of pyruvic acid involves the reduction of Lip (S–S) to Lip$\begin{matrix}\diagup\text{SH}\\ \diagdown\text{SH}\end{matrix}$ (oxidized and reduced lipoic acid, respectively) by pyruvate, and the regeneration of the disulfide by DPN or by an artificial oxidant, indophenol or ferricyanide. The proximity of the two thiol groups in lipoic acid permits the use of ferricyanide and indophenol, since the dithiol is oxidized to the required S–S form rather than over-oxidized to the level of sulfenic acid as probably would be the case if the coenzyme were a monothiol compound.

Preisler and Bateman[31] found that dithiobiuret is the only example of a thiol which undergoes a freely reversible oxidation–reduction. In the indophenol assay, this substance is oxidized only about 25% (50% on the basis of one thiol being oxidized) and at a very slow rate (2.3×10^{-3} μmole/ml./min.) The relative non-reactivity of this compound is due perhaps to a slow tautomerization, *i.e.*



which lowers the effective concentration of the free thiol.

Indophenol Assay Method for Thiols.—Various rapid, colorimetric methods have been used for the quantitative estimation of thiols. Most of these depend on the use of a chromophoric oxidant such as porphyrindin,[32] ferricyanide,[33] nitroprusside,[34] or phosphotungstate.[35] The information gained from the previous kinetic study of the indophenol–thiol reaction made it possible to devise a colorimetric assay based on this reaction.[36] A comparison of the indophenol assay method with the nitroprusside method of Grunert and Phillips[34] is shown in Fig. 4 where $\Delta \log I_0/I$ is plotted against concentration of cysteine. Both methods are accurate over

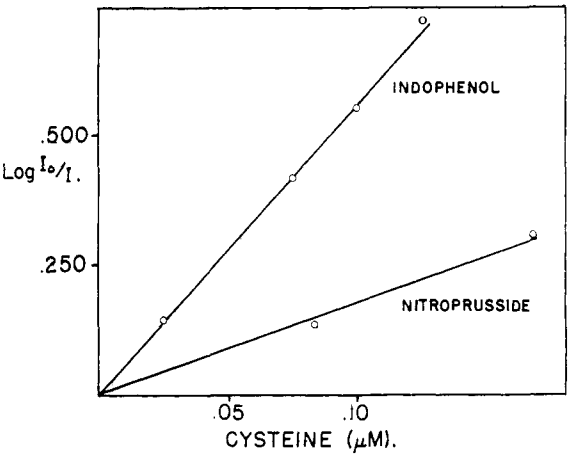


Fig. 4.—Comparison of indophenol and nitroprusside assay methods.

the same range of concentration (*ca.* 0.025 to 0.20 μmole). There are three advantages to the indophenol method: (a) The dye, which is bleached by the thiol compound, is not rapidly reoxidized so that the readings do not have to be taken rapidly after the addition of thiol as is the case with the nitroprusside method, due to the instability of the colored complex; (b) the indophenol method is slightly more sensitive, as can be seen from the figure, since there is a larger increment of color change per unit thiol added; and (c) the indophenol method will detect thiols in the presence of acyl thiol esters which are hydrolyzed under the basic conditions usually employed in the nitroprusside assay. The nitroprusside method has the advantage of being specific for thiols (and disulfides after reduction with cyanide) whereas other reducing substances (*e.g.*, ascorbic acid) interfere in the indophenol method and, hence, limit its use to purified thiols. Both assays suffer from the fact that the values obtained are not absolute, and must be compared with a *suitable* standard. That is, if an unknown compound is being assayed by either method, the results must be compared with a closely related, known compound, because of the many steric and structural effects on the reactivity of the thiol group in a molecule.

SEATTLE, WASHINGTON

(29) V. Jagannathan and R. Schweet, *J. Biol. Chem.*, **196**, 551 (1952).

(30) L. J. Reed and B. G. DeBusk, THIS JOURNAL, **75**, 1261 (1953); L. P. Hager and I. C. Gunsalus, *ibid.*, **75**, 3768 (1953).

(31) P. Preisler and M. M. Bateman, *ibid.*, **69**, 2632 (1947).

(32) J. P. Greenstein, *J. Biol. Chem.*, **125**, 501 (1938); **128**, 233 (1939).

(33) M. L. Anson, *J. Gen. Physiol.*, **22**, 247 (1939); **23**, 221 (1940); **25**, 355 (1942).

(34) R. R. Grunert and P. H. Phillips, *Arch. Biochem. Biophys.*, **30**, 217 (1951).

(35) J. J. Kolb and G. Toennies, *Anal. Chem.*, **24**, 1164 (1952).

(36) The indophenol method has been used by Mahler, *et al.* (*J. Biol. Chem.*, **210**, 465 (1954)) to estimate the –SH groups in aldehyde oxidase.