The Conjugation of Thiols by 2,6-Dichloroindophenol*

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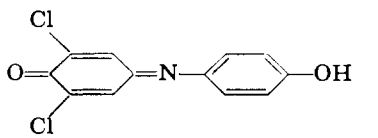
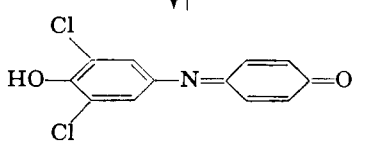
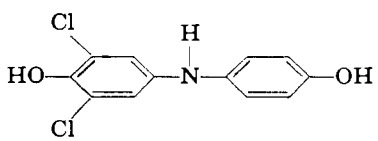
Previous work showed that when cysteine decolorized 2,6-dichloroindophenol, not only was the leuco dye formed but also an adduct between cysteine and the dye. The dye was capable of oxidizing the adduct. The oxidized adduct underwent further reaction with cysteine to yield a polysubstituted conjugate (*oxidative addition or oxidative substitution*). In contrast, while both glutathione and coenzyme A formed an adduct with the dye, the dye did not readily oxidize these adducts. These data suggested that glutathione might act at times as a nucleophilic scavenger in biological systems and thus protect vital sulfhydryl groups. NADH and NADHP reduced the dye without forming a conjugate. A variety of amino acids and nucleotides did not reduce or conjugate with the dye.

Recently, Hadler *et al.* (1963) found that the reaction between cysteine and 2,6-dichlorcindophenol involved more than simple oxidation reduction. Not only was the expected leuco dye, II, formed, but also a conjugate between the dye I and cysteine. The lower the original



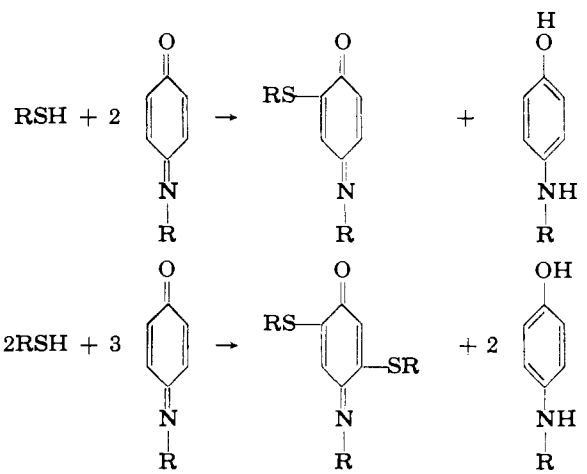


I



II

ratio of cysteine to dye the greater was the degree of substitution of the conjugated dye due to the increase in the number of addition-oxidation sequences. Such a process was designated *oxidative addition* or *oxidative substitution*. The over-all stoichiometries for oxidative substitution would be:



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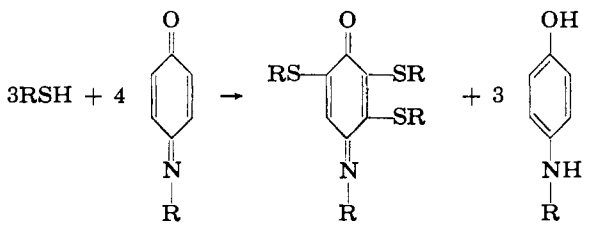

and so forth. In the present paper this study has been extended to include the two thiols glutathione and coenzyme A, and other nucleophiles from among the amino acids and nucleotides.

EXPERIMENTAL

Both coenzyme A and glutathione (Table I) were conjugated by the dye. At identical starting ratios of thiol to dye, the ratio of leuco dye to conjugate was significantly lower with coenzyme A and glutathione than with cysteine. In accordance with the proposed sequence of addition followed by oxidation (Hadler *et al.*, 1963), the conjugates formed from the reactions of coenzyme A and glutathione with the dye would consist mainly of the monosubstituted adducts in the reduced form as the ratios of leuco dye formed to conjugate were less than 0.5:1.

While the ratio of leuco dye to conjugate rose with increasing proportions of dye to glutathione (Fig. 1), the increase was less pronounced than with cysteine (Hadler *et al.*, 1963). With a 10-fold excess of dye to glutathione the ratio of leuco dye to conjugate reached the value of one. Even with an 8-fold excess of cysteine

TABLE I

THE CONJUGATION OF COENZYME A AND GLUTATHIONE

Each tube contained 200 μmoles of potassium phosphate adjusted to *p*H 6.5 with acetic acid and the designated amount of dye and thiol in a total volume of 6.0 ml. The procedure has been described (Fig. 6, Hadler *et al.*, 1963). The data with cysteine are from a previous experiment (Fig. 6, Hadler *et al.*, 1963) but are included for comparison.

	Co-enzyme A	Gluta-thione	Cys-teine
Ratio thiol to dye	2:1	2:1	2:1
Dye used (μmole)	0.318	0.321	0.320
Recovered dye (μmole)	0	0	0.013
Leuco dye (μmole)	0.049	0.088	0.179
Conjugate (μmole)	0.269	0.233	0.128
Ratio leuco dye to conjugate	0.18	0.38	1.40
Yield of conjugate, % of dye	85	73	40
Dye consumed, %	100	100	96

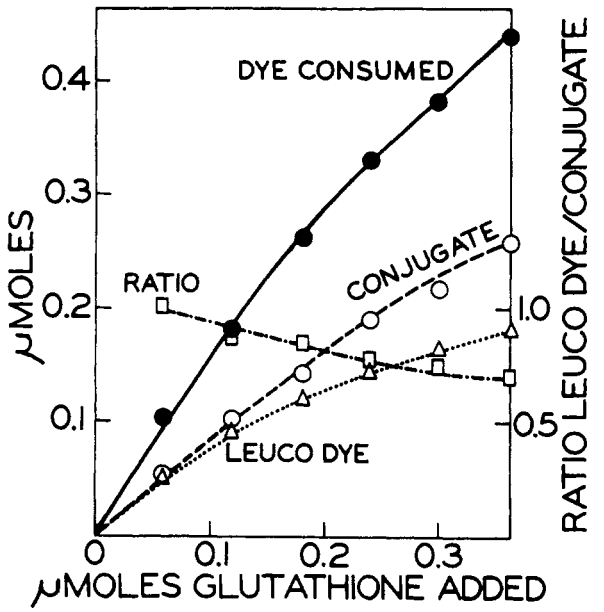

FIG. 1.—Variation of products from glutathione and dye. The procedure has been described (Fig. 6, Hadler *et al.*, 1963) except that each tube contained 0.6 μmole of dye.

to dye, the ratio of leuco dye to conjugate did not fall below one (Hadler *et al.*, 1963). Thus oxidative substitution was more difficult with glutathione than with cysteine.[1] Steric factors no doubt account for the resistance of the reduced monoadduct derived from glutathione to undergo oxidation. The large groups postulated to be in the side chain might readily hinder the approach of the oxidizing agent. The even lower ratio of leuco dye to conjugate found with coenzyme A (Table I) supports the suggested steric hindrance effect.

In addition to the steric effect the oxidizable hydrogens of an adduct, in the reduced form, might be stabilized by hydrogen bonding with a carbonyl function of a peptide linkage in the side chain. A ring incorporating such a hydrogen bond would have nine or ten members if a glutathione residue were involved. If a coenzyme A residue were involved, the ring would have ten or fourteen members. With a cysteine residue a nine-membered ring incorporating the carbonyl of the carboxyl group is possible.

When the thiol was glutathione, it was not possible to alter the ratio of leuco dye to conjugate by raising the temperature from 0° to 37° (Table II) or by using

TABLE II

THE EFFECT OF TEMPERATURE ON THE CONJUGATION OF GLUTATHIONE

The procedure has been described (Fig. 6, Hadler *et al.*, 1963). The incubations were carried out at the designated temperatures; each tube had 0.6 μmole of dye.

	Temperature			
	0°	37°	0°	37°
Ratio thiol to dye	0.25:1	0.25:1	0.5:1	0.5:1
Recovered dye (μmole)	0.417	0.422	0.256	0.274
Leuco dye (μmole)	0.083	0.085	0.145	0.134
Conjugate (μmole)	0.106	0.099	0.205	0.198
Ratio leuco dye to conjugate	0.78	0.86	0.71	0.68

[1] The data of Table I put coenzyme A in the same class as glutathione. Only glutathione was extensively studied.

TABLE III

ANAEROBIC CONJUGATION OF GLUTATHIONE

A three-necked flask was fitted with an alternating source of vacuum or nitrogen and a pressure-equalized dropping funnel. The dye and buffer were placed in the flask and the cysteine solution in the dropping funnel. The system was evacuated and filled with nitrogen three times and the cysteine solution quickly added and mixed with the flask contents. At the appropriate period of incubation a 6.0-ml aliquot was analyzed as described (Fig. 6, Hadler *et al.*, 1963). The reaction system was kept under positive nitrogen pressure. In each aliquot there was 0.312 μmole of dye. The starting ratio of glutathione to dye was 0.33:1.

	Period of Incubation (min)		
	20	40	60
Recovered dye (μmole)	0.149	0.147	0.149
Leuco dye (μmole)	0.074	0.074	0.074
Conjugate (μmole)	0.089	0.091	0.089
Ratio leuco dye to conjugate	0.83	0.82	0.83

anaerobic conditions (Table III). In a sequence of reactions where the oxidation of the reduced monoadduct by excess dye was difficult, auto-oxidation of the adduct conceivably might become significant and result in a fallaciously low leuco dye to conjugate ratio. The data obtained under anaerobic conditions eliminated this possibility.

A variety of nucleophilic groups in several amino acids, nucleotides, and NAD were unreactive.[2,3] The reactivity of the thiol group in coenzyme A was confirmed by the nonreactivity of the three adenine nucleotides.

While there was no conjugation with NADH, leuco dye was produced (Table IV). The molar ratio of NADH consumed and leuco dye produced was 1:1. Thus while the thiols and reduced dinucleotides examined were nucleophilic with respect to the dye, the moieties conjugated were RS^- and H^-, respectively.

TABLE IV

THE REACTIVITY AND NONCONJUGATION OF NADH

The procedure has been described (Fig. 6, Hadler *et al.*, 1963), except each tube contained 0.6 μmole of NADH and 0.3 μmole of dye, in a total volume of 6.0 ml. In another experiment the aqueous solution after extraction with ether was read at 340 mμ against a blank of buffer and water in order to include the disappearance of NADH. The ratio of leuco dye generated to NADH consumed was 1:1.

Time (min)	Oxidized Dye (%)	Leuco Dye (%)	Total (%)
10	92.2	8.9	101.1
20	82	15.2	97.2
30	79.5	22.8	102.3
40	72	27.7	99.7
50	60	36.3	96.3

[2] The abbreviations used are: NAD and NADH, respectively, nicotinamide adenine dinucleotide and its reduced form; NADHP, reduced nicotinamide adenine dinucleotide phosphate; TMP, 5′-phosphate deoxyribosyl thymine; UMP, 5′-phosphate ribosyl uracil.

[3] All the dye was recovered in the ether extracts after incubation with histidine, lysine, methionine, arginine, tryptophane, proline, tyrosine, glycine, serine, cystine, the three adenine nucleotides, UMP, TMP, NAD. In each experiment 0.6 μmole of the various compounds was incubated with 0.3 μmole of dye and analyzed as described (Fig. 6, Hadler *et al.*, 1963), except that for tyrosine and cystine the amounts were 2.0 and 1.1 μmoles, respectively.

TABLE V

REPRODUCIBILITY OF THE "MICRO" PROCEDURE

Each tube contained 40 μmoles of potassium phosphate adjusted to *p*H 6.5 with acetic acid, 0.12 μmole of dye, and the designated amount of cysteine in a total volume of 1.2 ml. Standards of oxidized and leuco dye (generated with 10 μmoles of potassium borohydride) were measured. The leuco dye standard was corrected for the slight absorbency of the potassium borohydride. After 20 minutes in ice the tubes were extracted with a single 4.0-ml portion of ethyl acetate (distilled analytical) using Pasteur pipets capped with a rubber bulb for mixing. After low speed centrifugation the extracts were read at 530 and 283 mμ against a blank of ethyl acetate.

Cysteine (μmole)	Leuco Dye (μmole)	Recovered Dye (μmole)	Conjugate (μmole)	Dye Consumed (μmole)	Ratio Leuco Dye/Conjugate
0.024	0.0263	0.088	0.006	0.0323	4.38
0.024	0.0268	0.081	0.012	0.0388	2.23
0.048	0.0510	0.0470	0.022	0.0730	2.32
0.048	0.0505	0.0466	0.022	0.0725	2.29
0.072	0.0645	0.0225	0.032	0.0965	2.01
0.072	0.0650	0.0250	0.030	0.0950	2.16
0.096	0.0720	0.00845	0.039	0.1110	1.85
0.096	0.0730	0.00845	0.039	0.1120	1.87

The scope of the experimental procedure was increased by replacing the three ether extractions by a single extraction with a smaller volume of less volatile ethyl acetate. The reproducibility was within 1% with as little as 0.05 μmole of cysteine (Table V).

The simplified procedure proved useful in obtaining extensive data from a single experiment (Fig. 2). As

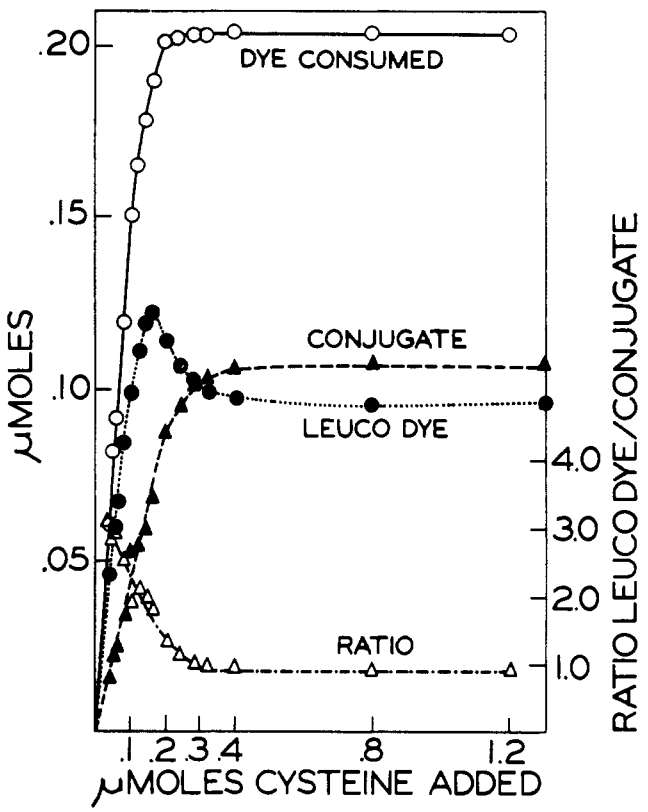

FIG. 2.—The scope of the conjugation of cysteine. The experimental procedure has been described (Table V). Each tube contained 0.2 μmole of dye, 60 μmoles of buffer, and the appropriate amount of cysteine in a total volume of 2.0 ml. 4.0 ml of ethyl acetate (distilled, analytical) was used for extraction.

TABLE VI

THE INFLUENCE OF *p*H ON THE CONJUGATION OF CYSTEINE

The procedure has been described (Table V). The volume of the reaction mixture was 7.2 ml. At the appropriate times 1.2-ml aliquots were analyzed. In each aliquot there was 0.12 μmole of dye, twice as much cysteine, and 40 μmole buffer.

Time (min)	Ratio Leuco Dye/Conjugate *p*H 6.5	*p*H 4.0
20	1.05	1.69
40	1.05	1.69
60	1.01	1.63

long as the starting ratio of dye to cysteine was two or more, the leuco dye generated was directly proportional to and equal to the starting cysteine. In this region of proportionality all the cysteine originally present participated in oxidative substitution. Also in this region the ratio of unconsumed dye to conjugate was one or more. In agreement with the concept of oxidative substitution (Hadler *et al.*, 1963), an excess of oxidizing agent over conjugated species was the critical requirement for linearity between added cysteine and leuco dye formation.

With a large excess of cysteine, half the dye was converted to leuco dye and half to conjugate (Fig. 2). The cysteine consumed by oxidative substitution equals the yield of leuco dye. Asymptotic conditions were attained with a 2:1 ratio of cysteine to dye. In this tube half the dye was conjugated, half the dye was converted to leuco dye, and one-fourth of the cysteine was conjugated by oxidative substitution.

At the end of linearity between the added cysteine and leuco dye generated (Fig. 2) there was 0.05 μmole of unreacted dye and 0.10 μmole of leuco dye. As the starting proportion of cysteine to dye is further increased, theoretically at least 0.025 μmole more of leuco dye might be generated. The maximum yield of leuco dye found was 0.122 μmole, a value consistent with the minimal theoretical sum of 0.10 μmole + 0.025 μmole. Another transition occurred in the region of maximum yield of leuco dye. Corresponding to a starting ratio of thiol to dye of 0.7:1 and 0.8:1, the ratio of cysteine not consumed by oxidative substitution to unreacted dye ranged from 0.021/0.025 to 0.038/0.013, respectively. The yield of leuco dye diminished sharply once the over-all conditions did not favor oxidative substitution.

The data do not indicate the fate of the cysteine not utilized in oxidative substitution. Neglecting autoxidation and self-condensation, at least two routes are open for further consumption of cysteine, without affecting the assayed yield of conjugate or leuco dye. The cysteine could be converted to cystine and the oxidized conjugate could become a reduced conjugate, or alternatively the cysteine could add 1,4 to an open position in a conjugate. A precedent for the first alternative has been provided by Schubert (1947) who previously had reduced the oxidized form of the completely substituted conjugate derived from benzoquinone and thioglycolic acid by an excess of thioglycolic acid.

When the *p*H was lowered to 4 (Table VI), there was a pronounced effect on the reaction between cysteine and the dye. The rate of dye consumption was lessened (Hadler *et al.*, 1963) but the ultimate ratio of leuco dye to conjugate was increased. Lowering the *p*H enhanced *oxidative substitution*. When cysteine was the thiol, the reactions following the formation of the reduced monoadduct were accelerated relative to the formation of the reduced monoadduct. In contrast,

TABLE VII

THE INFLUENCE OF *p*H ON THE CONJUGATION OF GLUTATHIONE

The procedure has been described (Table V). The ratio of glutathione to dye was 1:1.

Time (min)	Ratio Leuco Dye/Conjugate *p*H 6.5	*p*H 4.0
20	0.221	0.115
40	0.227	0.118
60	0.227	0.117

when glutathione was the thiol (Table VII), both the rate of dye consumption and the ratio of leuco dye to conjugate was decreased as the *p*H was lowered.

The observation that polysubstitution was favored by increased ratio of dye to cysteine was rationalized in the following manner. In a molecule with a system capable of undergoing reversible oxidation-reduction, the greater the number of electron donating substituents the lower the oxidation-reduction potential. Several references discussing this effect have been given (Hadler *et al.*, 1963). Thus the more substituted the reduced conjugate the greater the release of free energy on oxidation by the original dye. Opposing this enhancement of release of free energy will be the steric restrictions imposed when cysteine approaches an oxidized conjugate and when the dye approaches a reduced conjugate. Polysubstitution should also diminish the electrophilic nature of the oxidized conjugate, thus diminishing its energy of attraction for the nucleophile. Nevertheless when cysteine is the nucleophile the factors detrimental to polysubstitution are more than compensated by the oxidative release of free energy. With large thiols like glutathione and coenzyme A the steric factors became dominant, and in addition the reduced conjugates might be stabilized by hydrogen bonding.

The data presented assign three categories to the nucleophiles which react with 2,6-dichloroindophenol. Cysteine represents the first category of nucleophiles—compounds which readily undergo oxidative substitution. Glutathione and coenzyme A are members of a second class of nucleophiles—compounds which conjugate and participate reluctantly in oxidative substitution. The third class of nucleophiles is represented by NADH and NADPH. These nucleophiles yield the hydride ion as the conjugated residue.

The difference between cysteine and glutathione suggested a useful biochemical role for glutathione. As the amount of dye conjugated with glutathione was much greater than with cysteine (at a 2:1 ratio of thiol to dye the yield of dye conjugated was 73% and 40% with glutathione and cysteine, respectively, Table I), glutathione might protect sulfhydryl groups of an enzyme from an undesirable electrophilic reagent. In effect glutathione in some instances could serve as a *nucleophilic scavenger* for electrophiles alien to the biochemical system being protected.

Several observations in the literature are in keeping with such a role for glutathione. Haley and Robin (1962) found that increasing amounts of menadione (an electrophile) progressively lowered the level of glutathione in red blood cells. When a critical degree of glutathione depletion was reached further additions of menadione produced drastic irreversible changes in the erythrocytes. Nickerson *et al.* (1960, 1963) recently established the conjugation of menadione by glutathione.

The conjugation of thiols by 2,6-dichloroindophenol would render the dye useless as an oxidation-reduction indicator in a system where free thiols were present. Beinert (1962) pointed out that the dye could not be used for the assay of acyl coenzyme A dehydrogenase when free glutathione was liberated enzymatically.

ACKNOWLEDGMENT

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ADDED IN PROOF

Recently D. S. Coffey and L. Hellerman (1963, *Fed. Proc. 22*, 297) reported the conjugation of glutathione by indophenol.

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