Catalysis of Disulfide Interchange in Acid Media by Selenium and Tellurium Oxy Acids*

Paul J. Lawrence†

ABSTRACT: Sodium selenate, selenite, tellurate, and metavanadate catalyze the disulfide interchange reaction (RSSR + R′SSR′ = 2RSSR′) between L-cystine and *N,N′*-bis-2,4-dinitrophenyl-L-cystine in concentrated HCl solutions. The catalytic coefficients fall in the order $SeO_4^{2-} > SeO_3^{2-} > TeO_4^{2-} > VO_3^-$. This property is not shared by all of the group VIA oxy acids, and sodium sulfate, sulfite, tellurite, arsenite, phosphite, thiosulfate, and molybdate are inactive. Like the spontaneous interchange reaction, the selenite-catalyzed reaction is very sensitive to changes in acid concentration. The data suggest an initial critical protonation of the disulfide bond followed by reversible cleavage to form a highly reactive sulfenium ion and a strongly inhibitory thiol. Cysteine and iodide strongly inhibit selenate and selenite catalysis, but arsenite and tellurite exert no inhibition. Cysteine inhibition of selenate catalysis is a linear function of the cysteine concentration until the cysteine to selenate ratio is 4:1. The uncatalyzed reaction is first order with respect to reactant concentration but the selenite-catalyzed reaction has an order of 0.5 with respect to disulfide concentration. The known reactions between the selenium compounds and thiols suggest that they function catalytically by removing inhibitory thiols rather than by generating sulfenium ions.

In his early attempts to define the distribution of the disulfide bonds of insulin, Sanger (1953) hydrolyzed the hormone with concentrated HCl and found more disulfide peptide residues than would be expected from the known cysteine content of this protein. Model disulfides were used to demonstrate that disulfide interchange reactions can proceed in strongly acid media

$$RSSR + R'SSR' = 2R'SSR \qquad (I)$$

(Sanger, 1953; Ryle and Sanger, 1955). In neutral or in basic media, thiols catalyze disulfide interchange reactions and this effect is prevented by *N*-ethylmaleimide, by *p*-mercuribenzoate, or by molecular oxygen (Ryle and Sanger, 1955). Hence, in neutral or in basic media the reaction mechanism probably involves the attack of a mercaptide ion on a disulfide bond. In acid media, however, thiols inhibit interchange and Benesch and Benesch (1958) have suggested that the reaction in concentrated acid media involves the formation of highly reactive sulfenium ions, RS^+, which attack a disulfide bond. Sulfenyl chlorides catalyze the reaction, and it was shown that an aromatic sulfenyl chloride forms a sulfenium ion in acid media (Kharasch *et al.*, 1953). Several other organic catalysts were found and it was suggested that these also acted by generating sulfenium ions (Benesch and Benesch, 1958). In the present work, sodium selenate (Na_2SeO_4), sodium selenite (Na_2SeO_3), sodium tellurate (Na_2TeO_4), and sodium metavanadate ($NaVO_3$) were found to catalyze disulfide interchange in concentrated HCl. These compounds cannot serve as a direct source of sulfenium ions, and another mechanism must be proposed to explain their catalytic effect.

Methods and Materials

The system used to study disulfide interchange was that employed by Ryle and Sanger (1955) and Benesch and Benesch (1958), *i.e.*, the reaction between L-cystine and *N,N′*-bis-2,4-DNP-L-cystine. The colored interchange product, mono-DNP-cystine, unlike bis-DNP-cystine, is not extracted from an acid solution by diethyl ether. The extent of interchange was followed by measuring the absorption of the aqueous phase at 350 mμ after ether extraction. Solutions of sodium selenate and selenite in HCl slowly lost catalytic activity even when stored at −10°. Therefore, all such solutions were aqueous and interchange was initiated by adding the catalyst to the disulfide mixture in concentrated HCl. The extent of interchange occurring before addition of the catalyst was negligible. The loss of catalytic activity by solutions of the selenium compounds in HCl was probably a result of the reduction of these oxy acids to elemental selenium. None of the catalysts studied influenced the solubility of bis-DNP-cystine in diethyl ether.

Results

Catalysis by Sodium Selenate, Selenite, Tellurate, and

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† Present address: Laboratory for Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah, Salt Lake City, Utah 84112.

TABLE I: Specificity of Catalysis.[a]

Compound	Concn (M)	Time of Reaction (min)	Initial Rate of Interchange (% interchange/min)
H_2O		10	0.3
Na_2SeO_3	1.0×10^{-5}	10	35.0
Na_2SeO_3	1.0×10^{-6}	10	5.5
Na_2SeO_3	1.0×10^{-7}	120	0.3
Na_2SeO_4	1.0×10^{-5}	10	42.0
Na_2SeO_4	1.0×10^{-6}	10	20.5
$NaVO_3$	1.0×10^{-5}	10	9.0
$NaVO_3$	1.0×10^{-6}	10	0.4
Na_2TeO_4	1.0×10^{-6}	120	2.2
Na_2TeO_3	1.0×10^{-5}	120	0.3
Na_2SO_4	1.0×10^{-5}	120	0.3
Na_2SO_3	1.0×10^{-5}	120	0.2
$Na_2S_2O_3$	1.0×10^{-5}	120	0.2
$Na_2S_2O_4$	1.0×10^{-5}	120	0.5
Na_2SiO_3	1.0×10^{-5}	120	0.2
$NaHPO_3$	1.0×10^{-5}	120	0.4
$NaAsO_2$	1.0×10^{-5}	120	0.4
$NaMoO_4$	1.0×10^{-5}	120	0.2
NaN_3	1.0×10^{-5}	120	0.2
Methionine	1.0×10^{-5}	120	0.3
$CdCl_2$	1.0×10^{-5}	120	0.2

[a] Cystine, 1.0×10^{-3} M; bis-DNP-cystine, 1.0×10^{-4} M; HCl, 11.6 N; temperature, 30°. The initial rates of the reaction in the presence of catalyst were determined from the initial slopes of curves like those drawn in Figure 1.

Metavanadate.[1] Sodium selenate, sodium selenite, sodium tellurate, and sodium metavanadate are excellent catalysts of the disulfide interchange reaction in 11.6 N HCl. In the concentration range from 10^{-6} to 10^{-5} M, the order of catalytic activity is $SeO_4^{2-} > SeO_3^{2-} > TeO_4^{2-} > VO_3^-$ (Figure 1 and Table I). The extent of interchange at any given time in the presence of 1.0×10^{-6} M vanadate does not differ significantly from that of the uncatalyzed reaction. The order of catalytic activity is most apparent at low catalyst concentration, becoming less obvious as the catalyst concentration is increased.

Specificity of Catalysis. Whereas selenate, selenite, and tellurate catalyze the disulfide interchange reaction in strongly acid media, this capacity is not a property common to the group VIA oxy acids. Sulfite, sulfate, and tellurite are devoid of catalytic influence at 10^{-5} M. Also found inactive as catalysts were: cadmium chloride, methionine, sodium thiosulfate, hydrosulfite, metasilicate, molybdate, phosphite, arsenite, and azide (Table I).

[1] HCl catalyzes disulfide interchange, but the term "uncatalyzed reaction" will denote the reaction taking place in 4.8–11.6 N HCl.

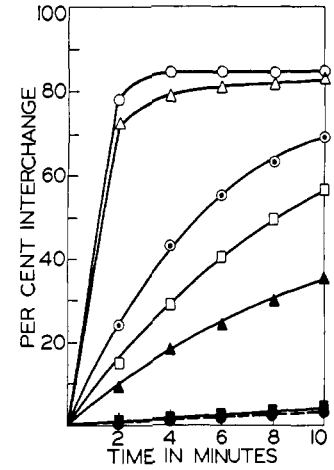

FIGURE 1: A comparison of the catalytic activities of sodium selenate, sodium selenite, and sodium metavanadate • bis-DNP-cystine, 1.0×10^{-4} M; cystine, 1.0×10^{-3} M; HCl, 11.6 N. (○) Sodium selenate, 1.0×10^{-5} M; (⊙) sodium selenate, 1.0×10^{-6} M; (△) sodium selenite, 1.0×10^{-5} M; (▲) sodium selenite, 1.0×10^{-6} M; (□) sodium metavanadate, 1.0×10^{-5} M; (■) sodium metavanadate, 1.0×10^{-6} M; (●) control; temperature, 30°. % interchange = (½)(concentration of mono-DNP-cystine at time t/initial concentration of bis-DNP-cystine) × 100.

Effect of Acid Concentration on Selenite Catalysis. Benesch and Benesch (1958) suggested that the sensitivity of the spontaneous interchange reaction to changes in acid concentration (Ryle and Sanger, 1955) is a result of a decrease in the rate of formation of sulfenium ions. The catalytic effect of sodium selenite demonstrates a similar dependence upon the acid concentration (Figure 2). At a selenite concentration of 1.0×10^{-5} M, a drop in the acid concentration from 11.3 to 10.1 N results in a considerable decrease in the extent of interchange at any time. The extent of interchange continues to decrease with a further drop in the acid concentration, and at 4.8 N acid, interchange proceeds to a very small extent in 10 min. The spontaneous interchange proceeds very slowly at pH 7.0 and the addition of 1.0×10^{-5} M selenite has no apparent catalytic effect over a 2-hr period. Selenite might be expected to inhibit the interchange reaction at this pH since it catalyzes the oxidation of thiols (Tsen and Tappel, 1958) but interchange proceeds very slowly in a neutral medium and inhibition could not be detected.

The acid sensitivity of the selenite-catalyzed interchange reaction is probably not a result of the degree of protonation of the selenite ion, since even in 4.8 N acid it exists largely as selenous acid ($K_1 = 2.4 \times 10^{-3}$; $K_2 = 4.8 \times 10^{-9}$) (Hagisawa, 1939). However, it is not unreasonable to postulate a protonation of the disulfide bond as a critical step in the over-all reaction (reaction

$$RSSR + H^+ = \overset{H}{[RSSR]^+} \qquad \text{(II)}$$

$$\overset{H}{[RSSR]^+} = RSH + RS^+ \qquad \text{(III)}$$

II). The heterolytic cleavage of the disulfide bond (reaction III) and the resulting formation of a sulfenium ion,

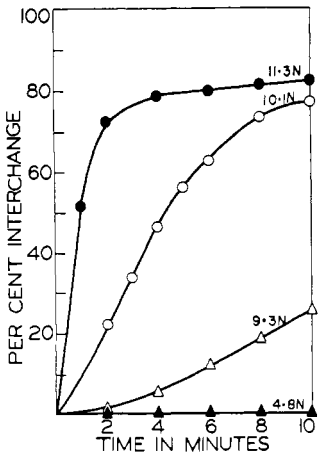

FIGURE 2: The effect of acid concentration on selenite catalysis of disulfide interchange. Bis-DNP-cystine, 1.0×10^{-4} M; cystine, 1.0×10^{-3} M; sodium selenite, 1.0×10^{-5} M; temperature, 30°. The HCl concentration is indicated above each curve.

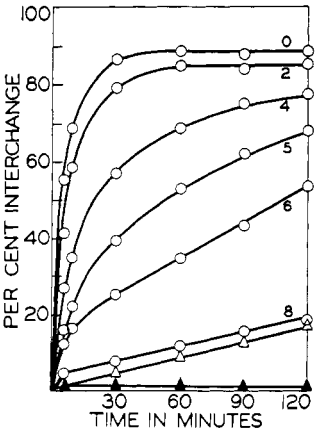

FIGURE 3: The influence of cysteine on the selenate-catalyzed interchange reaction. Bis-DNP-cystine, 1.0×10^{-4} M; cystine, 1.0×10^{-3} M; HCl, 11.6 N; sodium selenate, 1.0×10^{-6} M; temperature, 30°. (▲) Uncatalyzed reaction plus 1×10^{-6} M cysteine. (△) Uncatalyzed reaction. The number above each curve indicates the ratio of cysteine to selenate.

TABLE II: The Effect of Arsenite and Tellurite on Catalysis by Selenite.[a]

Time (min)	% Interchange			
	Selenite[b]	Selenite + Arsenite[c]	Selenite + Arsenite[d]	Selenite + Tellurite[e]
2	71.9	68.5	69.6	72.3
4	84.5	84.4	84.0	84.0
6	86.1	84.0	85.5	87.0
8	85.7	84.1	85.8	88.0
10	87.8	84.0	85.8	85.6

[a] Final concentrations: bis-DNP-cystine, 1.0×10^{-4} M; cystine, 1.0×10^{-3} M; HCl, 11.6 N; temperature, 30°. [b] Selenite concentration was 1.0×10^{-5} M. [c] 2.0×10^{-5} M sodium arsenite was added to the stock solutions of cystine and bis-DNP-cystine in concentrated HCl and allowed to incubate for 30 min. The reaction was started by adding sodium selenite after the disulfide solutions were mixed. The final concentration of sodium selenite and sodium arsenite was 1.0×10^{-5} M. [d] Aqueous equimolar solutions of arsenite and selenite were mixed and the resulting mixture was added immediately to the disulfide solution to initiate the exchange. The final concentration of each was 1.0×10^{-5} M. [e] Selenite and tellurite were mixed, and this mixture was immediately added to the disulfide solution to initiate interchange; the final concentration of each was 1.0×10^{-5} M.

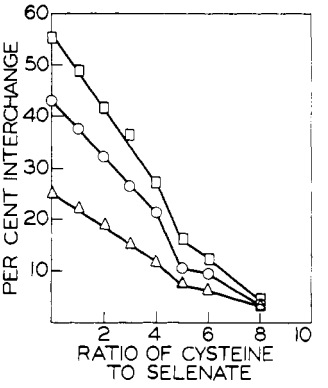

FIGURE 4: The influence of the cysteine: selenate ratio on the extent of interchange. Bis-DNP-cystine, 1.0×10^{-4} M; cystine, 1.0×10^{-3} M; HCl, 11.6 N; sodium selenate, 1.0×10^{-6} M; temperature, 30°. (□) Time, 6 min; (○) time, 4 min; (△) time, 2 min.

RS^+, resemble the acid-catalyzed formation of a carbonium ion, $\rangle C^+$, in many reactions of alcohols, *e.g.*, Wagner Meerwein rearrangement, pinacol rearrangement, etc. (Fieser and Fieser, 1961).

Inhibition of Catalysis. Tellurite and arsenite strongly inhibited the selenite-catalyzed oxidation of reduced glutathione at pH 7.1 (Tsen and Tappel, 1958). When arsenite was incubated for 30 min with reduced glutathione before addition of selenite, oxidation of reduced glutathione was inhibited, but if the arsenite and selenite were added simultaneously to the reduced glutathione solution no inhibition was observed. These authors suggested that "tellurite might compete directly with selenite," thereby preventing oxidation, and that arsenite forms a compound with reduced glutathione which inhibits oxidation. However, neither arsenite nor tellurite inhibits the selenite-catalyzed disulfide interchange, and incubation of the disulfides with arsenite before the addition of selenite has no effect on catalysis (Table II).

Although cysteine is a powerful inhibitor of the spontaneous and selenate-catalyzed interchange reaction in strongly acid media, its inhibitory effect is much less pronounced in the latter reaction. Whereas 1.0×10^{-6} M cysteine almost completely inhibits the spontaneous interchange reaction (Benesch and Benesch, 1958), its

effect on the selenate-catalyzed reaction is much less pronounced (Figure 3). Short-term experiments show that the extent of interchange in the selenate-catalyzed reaction is inversely proportional to the ratio of cysteine to selenate (Figure 4). The extent of interchange at any given time is a linear function of the cysteine to selenate ratio until the cysteine to selenate ratio reaches a value of four. At higher cysteine: selenate ratios there is a further decrease in the extent of interchange with a further addition of cysteine.

Potassium iodide strongly inhibits the spontaneous and the vanadate-catalyzed interchange reaction at 30° (Table III) as well as the selenite-catalyzed reaction

TABLE III: Inhibition of Spontaneous and Vanadate-Catalyzed Interchange by Potassium Iodide.[a]

Time (min)	% Interchange			
	H_2O	KI (1.0×10^{-5} M)	VO_3^- (1.0×10^{-5} M)	VO_3^- (1.0×10^{-5} M), KI (1.0×10^{-5} M)
30	5.07	0.658	67.5	10.2
60	12.3	2.76	74.5	20.4
90	14.3	4.60	78.6	29.3
120	18.0	5.25	80.0	37.0

[a] Bis-DNP-cystine, 1.0×10^{-4} M; cystine, 1.0×10^{-3} M; HCl, 11.6 N; temperature, 30°. Potassium iodide was introduced to the reaction medium 1 min before the addition of the catalyst.

at 0° (Figure 5). The temperature was lowered in the selenite experiments to prevent oxidation of the iodide. The influence of the inhibitor on the spontaneous interchange reaction was not determined at 0° since this reaction proceeds very slowly at lower temperatures. Iodide inhibition may be a result of a formation of inhibitory thiols *via* HI reduction

$$RSSR + 2HI = 2RSH + I_2 \qquad (IV)$$

Kinetic Considerations. The uncatalyzed reaction is first order with respect to bis-DNP-cystine concentration. Even after correcting the rate equation for the back-reaction (Laidler, 1963) a simple integral order with respect to reactant concentration could not be found using standard integral kinetics. Instead, the order of the reaction with respect to bis-DNP-cystine concentration was determined as follows (Laidler, 1963).

If a reaction has an order, by definition, the rate of the reaction, v, is related to the concentration of a given reactant by the equation

$$v = kc^n \qquad (V)$$

If the logarithm of this expression is taken

$$\log v = \log k + n \log c \qquad (VI)$$

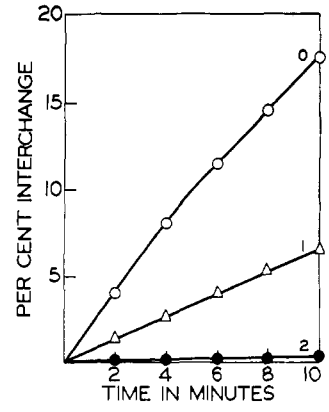

FIGURE 5: Iodide inhibition of selenite catalysis. Bis-DNP-cystine, 1.0×10^{-4} M; cystine, 1.0×10^{-3} M; HCl, 11.6 N; sodium selenite, 1.0×10^{-6} M; temperature, 0°. The ratio of KI to sodium selenite is shown above each curve.

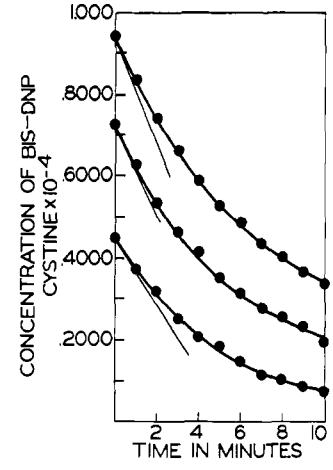

FIGURE 6: Disulfide interchange as a function of bis-DNP-cystine concentration. Cystine, 2.0×10^{-3} M; HCl, 11.6 N; sodium selenite, 1.0×10^{-6} M; initial concentrations of bis-DNP-cystine, 0.94×10^{-4}, 0.72×10^{-4}, and 0.45×10^{-4} M; temperature, 30°. The initial rate of interchange (straight lines) was determined by plotting the data on semilogarithm paper.

The order of the reaction may be determined by examining the initial rates of the reaction at various initial reactant concentrations. A plot of the logarithm of the initial velocity against the logarithm of the initial reactant concentration should give a straight line of slope n. Here n represents the order of the reaction with respect to the substance whose concentration was varied. This method has the advantage of providing an order which is not influenced by products, since all product concentrations are initially zero. This order has been called the *true order* or *order with respect to concentration* (Letort, 1937a,b, 1942).

Since cysteine, a product of the initial cleavage reaction, is a potent inhibitor of the interchange reaction, only the *true order* is significant. The order of the selenite-catalyzed reaction with respect to the bis-DNP-cystine concentration as determined by this procedure, subject to the inherent analytical errors, is 0.5 (Figures 6 and 7), indicating a complex reaction mechanism.

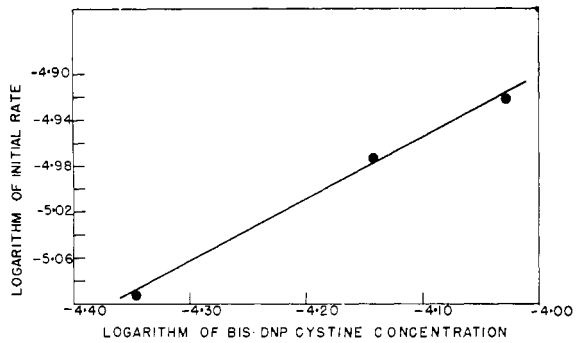

FIGURE 7: The determination of the order of the selenite-catalyzed interchange reaction with respect to bis-DNP-cystine concentration. The initial rates of the reaction were determined from the data obtained from Figure 6. The conditions of the reaction are listed with Figure 6.

Discussion

Early investigations into the mechanism of selenium poisoning revealed that glutathione is a detoxifying agent (DuBois *et al.*, 1939). Moreover, subcutaneous injection of Na_2SeO_3 into a rat appreciably lowers the levels of reduced glutathione in the blood, liver, and kidney. Suspecting that reduced glutathione was being bound and could not be measured as reduced glutathione, Lampson and Klug (1948) attempted to synthesize the compound which might be produced during detoxification. They found that 1 mole of selenous acid completely oxidized 4 moles of reduced glutathione, producing a substance with a sulfur to selenium ratio of 4:1. They believed that this compound was the glutathione analog of selenium tetracysteine prepared by Stekol (1942). Spectral and chemical evidence indicated that Stekol's selenium tetracysteine was, in reality, a mixture of cystine and selenium dicysteine (Klug and Petersen, 1949). Painter (1941) concluded that the reaction of cysteine and other thiols with selenous acid occurs as follows

$$4RSH + SeO_2 \rightarrow RSSR + RSSeSR + 2H_2O \quad \text{(VII)}$$

Petersen (1951) obtained data which seem to confirm this proposal.

The affinity of the selenium acids for thiols offers one possible explanation for their mode of action in the catalysis of a disulfide interchange reaction, *i.e.*, they may act as "antiinhibitors" by removing from the medium the thiols formed in the initial cleavage reaction. Schwarz and Sweeney (1964) and Cummins and Martin (1967) have also shown that sulfur compounds can bind selenite under acidic conditions, although the nature of the binding is unknown. As expected from the proposed mechanism, the differences in catalytic activity tend to decrease as the concentration of the catalyst increases (Figure 1, Table I).

This mechanism offers an explanation for the sensitivity of the selenite catalysis to changes in acid concentration (Figure 2). If the mechanism is valid, the inorganic catalyst cannot act until after heterolytic cleavage occurs (reactions II and III). This, in turn, is a function of the hydrogen ion concentration. The selenium acids in this mechanism would not function in the generation of RS^+ ions. Rather, their effect is largely a result of a removal of inhibitory thiols.

The failure of sulfite and tellurite to catalyze the disulfide interchange reaction may result from their inability to form the stable acids, H_2SO_3 and H_2TeO_3, respectively. Unlike SO_2, SeO_2 combines with water to form a definite compound, H_2SeO_3, which is stable in the crystalline state and in solution (Cotton and Wilkinson, 1962). The infrared spectrum of this compound confirms the existence of discrete H_2SeO_3 molecules (Falk and Giguère, 1958b). SO_2 is only moderately soluble in water and its solutions show no detectable amounts of H_2SO_3 (Falk and Giguère, 1958a; Jones and McLaren, 1958). It has been suggested that the greater polarity of the Se–O bond compared with the S–O bond may be responsible for these properties (Falk and Giguère, 1958b). Tellurium dioxide is also insoluble in water and it is doubtful that tellurous acid, H_2TeO_3, exists (Cotton and Wilkinson, 1962; Falk and Giguère, 1958b). Telluric acid, $Te(OH)_6$, however, does exist (Cotton and Wilkinson, 1962) and like selenous, H_2SeO_3, and selenic, H_2SeO_4, acid, it strongly catalyzes the disulfide interchange reaction in acid media.

The reactions may proceed as follows. First a protonated disulfide forms which can undergo cleavage to form a highly reactive RS^+ moiety and an inhibitory thiol. In the absence of catalyst, the back-reaction is sufficiently rapid to keep RS^+ at a very low steady-state concentration. In the presence of catalyst, the RSH may be consumed and the interchange may proceed. The actual products of the reaction between the selenium acids and thiols are not known. If it is assumed that selenium acids remove inhibitory thiols from the reaction scheme, a complex rate equation can be derived which correctly predicts the order with respect to disulfide concentration.

Any agent capable of removing thiols from the acid media should, according to this mechanism, catalyze the interchange reaction. Heavy metal sulfide precipitants and other thiol agents which depend upon the existence of free mercaptide ion, RS^-, would probably be inactive, since any sulfhydryl group would surely exist almost exclusively in the un-ionized form, RSH. Presumably, this explains the inability of arsenite to influence the rate of the selenite-catalyzed reaction in strongly acid media (Table II).

This type mechanism might also be examined in a previously cited example of catalysis observed by Benesch and Benesch (1958). A catalytic effect was observed when a conjugate which forms when the dye 2,6-dichloroindophenol reacts with cysteine was added to the disulfide interchange mixture. Catalysis was attributed to the generation of RS^+ ions by the conjugate, although its identity was unknown at the time. The conjugate was studied by Hadler *et al.* (1963), and the term *oxidative substitution* was used to describe its formation. This term emphasized that the dye, a *p*-quinone-like compound, must be in the oxidized state before substitution can occur. Contrary to expectations, polysubstitution of the dye by cysteine was favored

when the dye was present in a large excess. Hadler *et al.* (1963) explained this as follows. When an electron-releasing substituent adds 1,4 to a quinone, a substituted hydroquinone forms. This substituted hydroquinone is the reduced component of a couple whose oxidation–reduction potential is lower than that of the parent unsubstituted quinone. Hence, the substituted hydroquinone may be oxidized to a substituted quinone by the unsubstituted quinone. Several groups may be added to a quinone by a repetition of this process. Moreover, polysubstitution of 2,6-dichloroindophenol by cysteine is enhanced by lowering the pH. The conditions used by Benesch and Benesch (1958) to prepare the conjugate (a 1:1 ratio of cysteine to dye at pH 7.0) did not favor polysubstitution. The conjugate thus formed was capable of further substitution by cysteine. The long duration of their experiment (4 hr) permitted air oxidation of the conjugate. This oxidation would be necessary to permit a further substitution of the dye, and Hadler *et al.* (1963) found that the unsubstituted dye undergoes air oxidation during a 15-min spectral scan. Thus, rather than generating RS^+ ions as postulated by Benesch and Benesch (1958), the conjugate may function as a catalyst *via* RSH removal. Significantly, the conjugate formed at pH 4 is devoid of catalytic activity in the disulfide interchange system (Benesch and Benesch, 1958), although Hadler and Erwin (1963) has shown that *oxidative substitution* of the dye by cysteine is enhanced under these conditions.

Acknowledgments

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