Studies on Quinone-Thioethers. I. Mechanism of Formation and Properties of Thiodione*

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Reaction of 2-substituted 1,4-naphthoquinones with glutathione yields derivatives containing a glutathionyl group bound to the 3-ring carbon *via* the sulfur of glutathione. Yield and rate of thioether formation depend upon the electron-withdrawing ability of any substituent on the quinone ring. The reaction is shown to be a nucleophilic substitution. The 3-glutathionyl derivative of 2-methyl-1,4-naphthoquinone, termed thiodione, and its water-soluble sodium salt were isolated. In contrast to the mechanism postulated by other workers for addition of thiols to quinones, it is shown that oxidized thio-substituted quinone is formed without prior accumulation of thio-substituted hydroquinone. Hydrogen eliminated upon bond formation between sulfur and a ring carbon is taken up by a second molecule of quinone.

The addition of thiols to quinones was first studied by Bongartz (1888), who obtained hydroquinone-thioglycolic acid upon reacting benzoquinone with thioglycolic acid. Snell and Weissberger (1939) found that this reaction resulted in substituted hydroquinone when excess thiol was used, but gave the oxidized species when excess benzoquinone was present initially. They postulated the formation of substituted hydroquinone as the primary reaction product, and subsequent oxidation thereof by excess quinone. The same reaction sequence was postulated by Schubert (1947) for the addition of thioglycolic acid to benzoquinone, and by Fieser and Turner (1947) for that of various sulfhydryl compounds to menadione (2-methyl-1,4-naphthoquinone). In no instance, however, did these workers demonstrate the formation of reduced quinone-thioether during the course of the reaction. Formation of a complex between menadione and glutathione was indicated by Fieser and Fieser (1956). This complex, which we have termed thiodione (Nickerson *et al.*, 1960), has been prepared in this laboratory, and the mechanism of its formation has been elucidated.

EXPERIMENTAL

Materials.—*p*-Benzoquinone and 1,4-naphthoquinone and its 2,3-dichloro derivative were obtained from Eastman Organic Chemicals. Thioglycolic acid, menadione, and phthiocol were obtained from Mann Research Laboratories. All of the quinones were recrystallized from alcohol. Glutathione and glutathione monosodium salt were obtained from Schwarz Bio-Research, Inc. All solutions were freshly prepared for each experiment and were kept in the dark.

Anaerobic Reactions.—To free solutions of dissolved oxygen, they were evacuated in a closed system, boiled at room temperature, and flushed twice with purified nitrogen. Absorption spectra were determined in a demountable 1-cm quartz cuvet (Fig. 1)[1] which could similarly be evacuated and flushed with nitrogen.

Palladium Reduction.—Reduced forms of menadione and of thiodione were prepared in solution by use of hydrogen in the presence of finely divided palladium.

Absorption spectra of reduced materials were obtained after removal of palladium by centrifugation and immediate transferral of the clear supernatant to absorption cells which were then tightly stoppered. No detectable reoxidation by air was observed under these conditions. Spectra were measured in a Beckman DU or a Cary Model 14 spectrophotometer.

Redox Potentials.—To establish reduction potentials, solutions of the various quinones and complexes were titrated with titanous chloride, following the procedure of Michaelis *et al.* (1936). All solutions were prepared in 70% ethanol (final concentration) and contained 0.1 M each of HCl and KCl. Solutions of complexes were 1.5×10^{-4} M; $TiCl_3$ was obtained as a 20% solution which was diluted to approximately 3×10^{-3} M in 70% ethanol. Potential change during titration was measured by means of a Beckman Model G *p*H meter (used as potentiometer) fitted with platinum and saturated calomel electrodes. To accelerate equilibration of the electrodes, titrations were carried out in a water bath at 40°.

Detection of Reduced Menadione.—To 0.5 ml of anthranilic acid solution (4×10^{-3} M in 95% ethanol), 1.0 ml of 1 N HCl was added and the solution was cooled in an ice bath; 0.5 ml of aqueous sodium nitrite solution (1.2×10^{-2} M) was added, mixed, and allowed to react for 3 minutes at 0°. This was followed by 0.5 ml of aqueous ammonium sulfamate solution (2.4×10^{-2} M). After 2 minutes, 1.3 ml of 1 N Na_2CO_3 (ice cold) was added to yield a *p*H of 9.0–9.5, and followed immediately by 1.0 ml of test solution. In the presence of reduced menadione, a color developed immediately; oxidized menadione did not react in this test.

Since color development was essentially instantaneous at 0° and no evidence of gas evolution was discerned (albeit the concentration of reactants was low), we assume that the reaction involved a coupling of an ionized hydroquinone (analogous to that of a phenolate ion) with a diazonium chloride. This view is strengthened by the fact that no color change was observed with reduced thiodione (in which no free *ortho* position is available).

Determination of Sulfhydryl Groups.—Free SH groups were determined by titrating the sample with phenylmercuric nitrate, with nitroprusside as indicator. This is a modification (Powning and Irzykiewicz, 1960) of the method of Katchalski *et al.* (1957).

Determination of Bound Water.—A 5-ml volumetric pipet with tip sealed and wide end cut short was used. A sample of crystalline thiodione, previously dried *in vacuo* over soda-lime, was introduced into the bulb of the pipet through the short end, which then was

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[1] Assembly manufactured by Quaracell Products, Inc., 366 Broadway, New York 13.

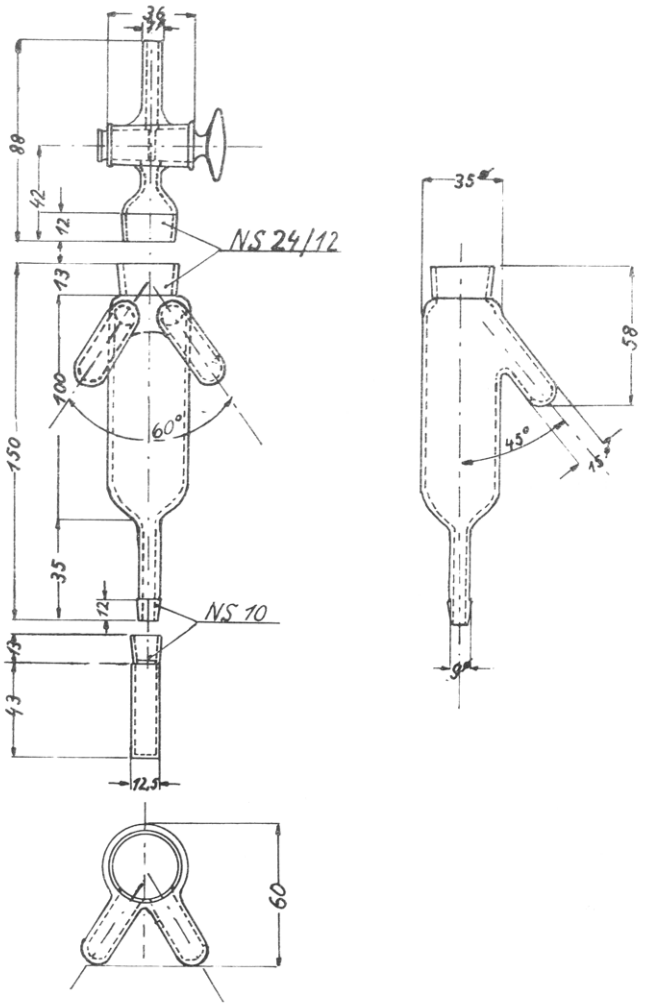


FIG. 1.—Evacuable quartz cuvet employed in spectral studies under anaerobic conditions. In use, the cell was tilted to an almost horizontal position; 3 ml of test solution was flowed into the upper glass reservoir and boiled under vacuum; the cell was then turned upright and filled with purified nitrogen until atmospheric pressure was restored.

sealed. In this way, the section of the pipet between bulb and tip was kept free of adhering sample. The bulb containing the sample was placed vertically into an oil heating bath; the protruding tube passed through an asbestos heat shield and was kept cool by a stream of air. On slow heating, neither evolution of water nor any change in the sample was observed up to 145°. At this temperature colorless droplets, identified as water (m.p., b.p., and Karl Fischer titration), began to condense in the cooled tube. The temperature was maintained at 150° for 30 minutes. After cooling, the tube was broken off, immediately sealed by a rubber stopper, weighed, rinsed with methanol, dried, and reweighed.

Paper Chromatography.—To identify reaction products and to estimate amounts of unreacted materials, reaction mixtures were subjected to paper chromatography. A satisfactory separation was achieved in an isopropanol – formic acid – water (70:10:20) system. Spots were first located by inspection under ultraviolet light (about 360 mμ). Chromatograms were then sprayed with 0.1% ninhydrin in water-saturated butanol and developed by heat (5 minutes at 90°). Spots due to glutathione and to complexes containing glutathione were ninhydrin positive and developed a color, but ultraviolet-absorbing spots due to unreacted quinone were ninhydrin negative.

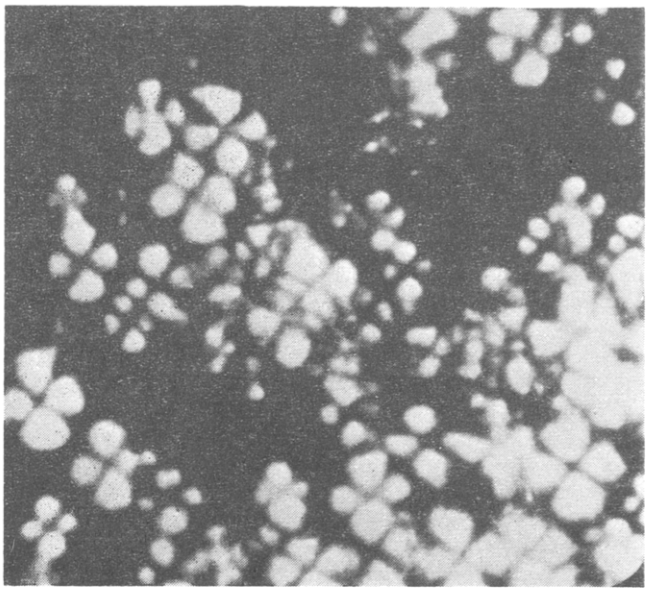


FIG. 2.—Crystals of thiodione as seen under crossed polarizers. Magnification 650×.

Preparation of Thiodione.—Twenty mmoles of recrystallized menadione were dissolved in 210 ml of 95% ethanol. To this was added 20 mmoles of reduced glutathione dissolved in 70 ml water. A dark color developed rapidly after mixing of the solutions. After the solutions had stood overnight at 4°, a crop of dark brown crystals formed, which was collected by filtration, washed with 95% ethanol, and dried at room temperature *in vacuo* over soda-lime; yield, 80% of theory for the aerobic reaction. A subsequent crop (12.5% of theory) was obtained from the filtrate on standing. The product was recrystallized by dissolving 1 g in 50 ml water on heating, adding 100 ml of 95% ethanol, and allowing to stand at room temperature.

Anal.[2] 2 - Methyl - 3 - glutathionyl - 1,4 - naphthoquinone monohydrate: Calcd. for $C_{21}H_{25}N_3O_9S$: C, 50.90; H, 5.09; N, 8.48; S, 6.47. Found: C, 51.32; H, 5.58; N, 8.50; S, 6.54. Calcd. for $C_{21}H_{23}N_3O_8S \cdot H_2O$: H_2O, 3.62. Found: H_2O, 3.45.

Preparation of Thiodione Sodium Salt.—Two mmoles of 2-methyl-1,4-naphthoquinone were dissolved in 10 ml of 95% ethanol; this solution was added immediately to a solution of 1 mmole of reduced glutathione monosidum salt in 10 ml of 60% ethanol. The mixture was kept overnight at room temperature or at 37°. At the end of this time a precipitate had formed. Additional precipitate was formed upon cooling of the mixture to −10°. The precipitate was filtered off, washed exhaustively with acetone, redissolved in water, and reprecipitated with acetone. The compound thus obtained was completely free of quinone and of reduced glutathione but contained traces of oxidized glutathione carried over from traces present in the starting material.

Final purification was achieved by passing a solution of monosodium thiodione through a cellulose chromatographic column. The cellulose was prepared by the method of Whitby (1952), and a mixture of isopropanol–formic acid–water (70:10:20) was employed for development of the column. Fractions containing pure thiodione were concentrated in a flash evaporator under vacuum and subsequently lyophilized. The lyophilized product was dissolved in water. Acetone was slowly added with stirring until a faint turbidity appeared. On standing at room temperature, crystals (yield, 80%

[2] Analyses by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

TABLE I
REACTIONS BETWEEN 1,4-NAPHTHOQUINONES AND MONOSODIUM GLUTATHIONE

Substituents on Naphthoquinone	Reaction Rate	Color of Product(s)[a]	R_F of Product(s)[b]
None	Slow	Yellowish-brown (mono G)	0.65 (major)
		Yellowish-brown (di G)	0.35 (minor)
2-Methyl (menadione)	Slow	Yellow (thiodione)	0.70
2-Methyl-3-hydroxy (phthiocol)	No reaction	—	—
2,3-Dichloro	Fast	Brownish-yellow (mono G)	0.71 (minor)
		Orange (di G)	0.31 (major)

[a] Mono G and di G refer to monoglutathionyl and diglutathionyl substitution, respectively. [b] R_F determined in isopropanol–formic acid–water (70:10:20) system.

of theory) developed and were collected; the yellow product, m.p. 198–201° with decomposition, was readily soluble in water but insoluble in acetone, ethanol, ether, or hexane. The quadrilaterally symmetrical crystals are shown in Figure 2.

Anal.[1] 2 - Methyl - 3 - glutathionyl - 1,4 - naphthoquinone monosodium salt monohydrate: Calcd. for $C_{21}H_{24}N_3O_9SNa$: C, 48.74; H, 4.67; N, 8.12; S, 6.20; Na, 4.44. Found: C, 48.61; H, 4.67; N, 8.14; S, 6.16; Na, 4.91.

RESULTS

Reactions.—To study the reactivity of substituted naphthoquinones with monosodium glutathione, solutions of variously substituted 1,4-naphthoquinones and monosodium glutathione were mixed in 70% ethanol. Reaction products which precipitated after various time intervals were isolated, purified, and examined by paper chromatography. Characteristics of the products obtained in presence of air are given in Table I.

In view of the fact that only one product was obtained on reaction of monosodium glutathione with 2-methyl-1,4-naphthoquinone and no product was formed with 2 - methyl - 3 - hydroxy - 1,4 - naphthoquinone, whereas two products were obtained with both unsubstituted naphthoquinone and 2,3-dichloro-1,4-naphthoquinone, it may be concluded that monosodium glutathione can add to positions 2 and 3 of the quinone ring. The products obtained represent complexes containing one or two glutathionyl groups.

To study further the relative ease of forming mono- and di-substituted products, 1 mmole dichloronaphthoquinone was reacted aerobically with various amounts (0.5–2.0 mmoles) of monosodium glutathione. The relative amounts of mono- (R_F 0.71) and di-substituted (R_F 0.31) product were estimated from chromatograms of each reaction mixture (Table II). Only two products were detected chromatographically. Approximately equal amounts of mono- and di-substituted product were obtained even at the lowest ratio of monosodium glutathione to dichloronaphthoquinone, whereas at the highest ratio employed, the di-substituted product predominated.

TABLE II
REACTION OF 2,3-DICHLORO-1,4-NAPHTHOQUINONE (DCN) WITH GLUTATHIONE

mmoles Glutathione/mmole DCN	Relative Amounts of Substituted Product		Free DCN in Mother Liquor	Total Product (mg)
	mono	di		
0.5	+	+	+	182
1.0	+	+ ±	+	360
1.5	+	+ +	±	526
2.0	±	+ + +	±	650

Mechanism of Thiodione Formation.—In the course of the reaction between menadione and monosodium glutathione, the concentration of free SH^- decreased with time. The rate of disappearance of SH^- was the same in the presence or absence of air, at least during the early stages of the reaction, and during this period was first order with respect to SH^-. At various times during the reaction, the concentration of thiodione (ϵ_{420} = 1170) was determined spectrophotometrically after correction for unreacted menadione (ϵ_{420} = 57). Concentration of the latter was calculated on the assumption that the loss in the menadione at any time equalled the decrease in SH^-. The amounts of thiodione formed and of SH^- lost (measured independently) were in good agreement at all stages of the reaction (Fig. 3). Therefore, glutathione may be presumed to attach to the quinone ring *via* its sulfhydryl group. On admission of air to a partially reacted mixture, a small increase in optical density developed immediately. Thus, in addition to a colored product, a colorless species, which turned yellow on oxidation, was also formed during the reaction. Presumably, this substance is a reduced quinone.

To confirm this supposition, a test for reduced quinone was developed. The test was negative with solutions of oxidized menadione or thiodione and positive with the reduced forms (obtained by reduction with

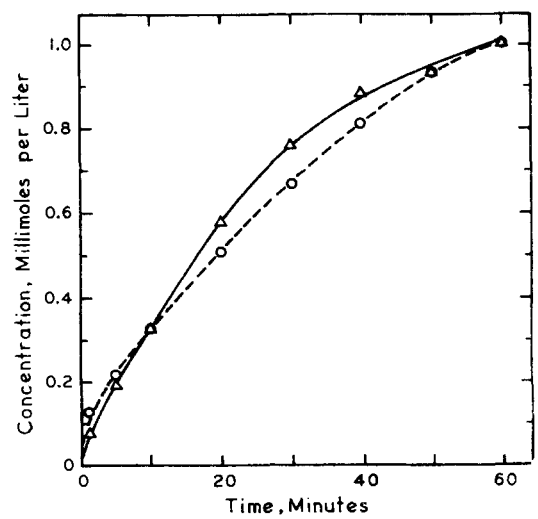


FIG. 3.—Changes in sulfhydryl and thiodione concentrations during aerobic reaction of 2.33×10^{-3} M menadione with 1.67×10^{-3} M monosodium glutathione in 70% ethanol. Δ, decrease in SH concentration (by titration with phenyl mercuric nitrate). O, concentration of thiodione (calculated from measured optical density at 420 mμ, after correction for unreacted menadione, as described in text).

TABLE III

IDENTIFICATION OF COLORLESS REACTION PRODUCT AS REDUCED MENADIONE BY CHANGE IN OPTICAL DENSITY ON AERATION

Time (min.)	Observed OD at 420 mμ	Molarity of Thiodione × 10^3	Molarity × 10^3 of Reduced Menadione	Molarity × 10^3 of Oxidized Menadione	Calculated OD
0	0.180	0	0	3.18	
18	0.775	0.56[a]	0.56[b]	2.06[b]	
18 + air	0.825	0.56	0	2.62	0.805[c]

[a] Molarity of thiodione formed (= x) calculated from observed OD by assuming that 2 moles of menadione (ϵ_{420} = 56.6) reacting anaerobically with glutathione produce 1 mole of reduced menadione (ϵ_{420} = 0) and 1 mole of thiodione (ϵ_{420} = 1170): $0.775 = (3.18 \times 10^{-3} - 2x)\,56.6 + 1170\,x$, from which $x = 0.56 \times 10^{-3}$ M. [b] Basis of calculation: Reduced menadione = thiodione; residual oxidized menadione = original menadione − (thiodione + reduced menadione) = $(3.18 - 2 \times 0.56)\,10^{-3} = 2.06 \times 10^{-3}$ M. [c] Calculated OD = $0.56 \times 10^{-3} \times 1170 + 2.62 \times 10^{-3} \times 56.6 = 0.805$, a value within 3% of the observed OD.

TABLE IV

YIELD OF THIODIONE IN ABSENCE AND PRESENCE OF AIR

Condition	Original Molarity × 10^4 of Menadione	Original Molarity × 10^4 of Glutathione	OD After 48 Hr.	Molarity × 10^4 of Thiodione Observed from OD[a]	Molarity × 10^4 of Thiodione Theoretical	Yield (% of theory)
N_2	16.7	16.7	0.730	4.05	16.7	24.2
N_2	8.3	8.3	0.480	2.67	8.3	32.2
Air	16.7	16.7	1.600	8.88	16.7	53.0
Air	8.3	8.3	0.830	4.61	8.3	55.5

[a] Corrected for absorbance due to unreacted menadione.

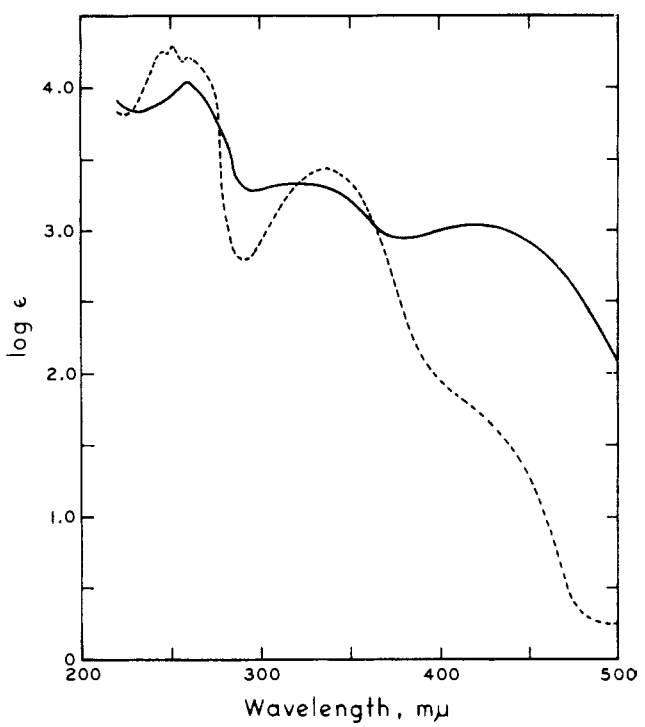


FIG. 4.—Molar absorption coefficients (cm^2 $mole^{-1}$) of thiodone (———) and menadione (– – – –) in 70% ethanol.

hydrogen over palladium) of either of these substances. A mixture of menadione and glutathione in 80% ethanol gave a positive test for reduced quinone, despite the presence of dissolved air. Samples taken at intervals showed that reduced quinone was still present 30 minutes after the start of the reaction but disappeared on longer standing.

To determine whether menadione or thiodione supplied the reduced species, advantage was taken of the marked difference in their absorption at 420 mμ. At this wave length the molar extinction coefficients are 56.6 (menadione) and 1170 (thiodione), as shown in Figure 4. From the magnitude of increase in optical density upon admission of air (Table III), it can be deduced that the colorless species initially formed was reduced menadione.

Reaction mixtures of equimolar amounts of menadione and monosodium glutathione were allowed to stand in the presence or absence of air. Optical density readings made at various time intervals showed that equilibrium had been reached after 48 hours. Aerobically, the yield of thiodione at equilibrium was twice as great as that obtained anaerobically (Table IV), since reduced menadione is unreactive toward glutathione. Indeed, no thiodione was formed on mixing monosodium glutathione with menadione that had been reduced with hydrogen and palladium. However, on aeration of this mixture, a yield of thiodione equal to that resulting from corresponding amounts of oxidized menadione and glutathione was formed (Table V).

Reduction of Thiodione by Excess Glutathione.—Thiodione was found to be slowly reducible by monosodium glutathione; only 40% of the thiodione was reduced when a 3×10^{-3} M solution was reacted anaerobically for one hour at room temperature with a 100-fold excess of monosodium glutathione. When 1×10^{-3} M menadione was reacted anaerobically with 2×10^{-2} M monosodium glutathione at *p*H 5 in 70%

TABLE V

THIODIONE FORMATION ON REACTION OF GLUTATHIONE WITH REDUCED AND OXIDIZED MENADIONE

(All concentrations initially 2×10^{-3} M in 80% ethanol)

Elapsed Time	Optical Density: Reduced Menadione	Optical Density: Oxidized Menadione
0	0.040	—
5 min.	0.041	0.325
10 min.	0.043	0.400
15 min.	0.047	0.470
18 hr.	1.84[a]	1.82[a]

[a] Both solutions exposed to air after 15 min. and left in contact with air for 18 hr.

ethanol, the initial rate of thiodione formation (as measured by optical density at 420 mμ) was 76% of that observed aerobically under comparable conditions. The concentration of thiodione became constant after 45 minutes of reaction and thereafter declined very slowly. Therefore, reduction of thiodione by monosodium glutathione becomes important only in the presence of a large excess of monosodium glutathione. The increase in optical density from the commencement of the reaction (Fig. 5) demonstrates that, even with a 20-fold excess of monosodium glutathione and in the absence of air, the initial product was thiodione, not the reduced thioether.

Change in Redox Potential Accompanying Thioether Formation.—The reaction between menadione and monosodium glutathione was carried out in a special evacuable cell, fitted with platinum and saturated calomel electrodes (Strauss and Nickerson, 1961), which permitted solutions contained therein to be degassed under vacuum and to be equilibrated with purified nitrogen at atmospheric pressure. Crystals of menadione (0.2 mmole) were suspended in 60 ml of an aqueous solution of 0.1 mmole of monosodium glutathione buffered with 0.05 M Tris at *p*H 7.3. Potentials were read at frequent intervals while the solution was agitated vigorously with a magnetic stirrer. The menadione gradually dissolved, and a deep yellow color developed in the solution. The potential fell rapidly from an initial value of +0.132 v (relative to the normal hydrogen electrode) to a final value of −0.035 v, which was reached in 4 hours. At that time air was bubbled through the solution, and the potential rose to +0.134 v within 2 minutes, with no apparent change in color. Absence of a significant color change upon reoxidation constitutes further evidence that the oxidation product was menadione (the molar extinction coefficient of which is only one twentieth that of thiodione).

Redox Potentials of 1,4-Naphthoquinones.—Potentiometric titration of monosodium thiodione with $TiCl_3$ at *p*H 1.0, as described under Methods, gave a redox potential of +0.335 ± 0.002 v, corresponding to an E_0 (at *p*H 0) of +0.395 v in 70% ethanol solution. Similar E_0 values for monosodium thiodione were obtained by titration with $TiCl_3$ in *p*H 4.0 acetate buffer and by oxidative back-titration with iodine. Titrations with sodium dithionite gave erratic results, probably owing the formation of bisulfite addition compounds. Autoxidation of reduced thiodione by air rapidly went to completion (Fig. 6).

Menadione, when titrated with $TiCl_3$ at *p*H 1.0 in 70% ethanol, gave an E_0 of +0.382 v. E_0 values for menadione reported in the literature range from 0.422 to 0.408 v and appear to depend on the solvent used (Clark, 1960). The redox potentials of thiodione and menadione reported here are to be regarded as comparative rather than absolute, since they involve uncertainties in the potential of the calomel electrode in alcoholic solution and in the actual *p*H of the alcoholic solutions. Since menadione has a slightly lower redox potential than thiodione, the possibility of interaction between reduced menadione and thiodione during formation of the latter compound had to be considered. A stoichiometric amount of $TiCl_3$ was added to a 1×10^{-3} M solution of menadione at *p*H 1.0 in 70% ethanol, after which excess monosodium thiodione was added anaerobically. The potential, which had fallen to +0.210 v at the end of the reduction step, did not rise beyond +0.290 v (a value well below the redox potential of +0.322 v for menadione at *p*H 1), even on standing for 6 hours. Therefore, thiodione does not oxidize reduced menadione. The opposite reaction, namely, oxidation of reduced thiodione by menadione,

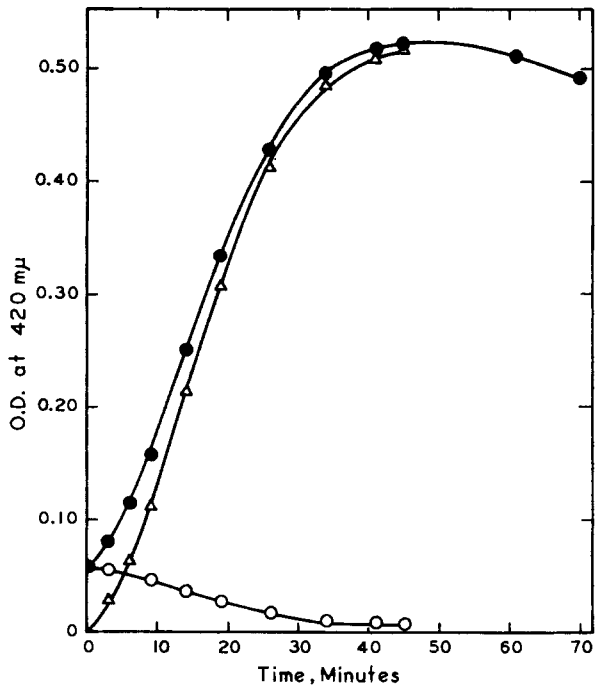


FIG. 5.—Formation of thiodione during anaerobic reaction of menadione (1×10^{-3} M) with a large excess of monosodium glutathione (2×10^{-2} M) in 70% ethanol. ●, observed optical density at 420 mμ. O, OD due to unreacted menadione (calculated on the assumption that two moles of menadione are consumed per mole of thiodione formed). △, OD due to thiodione (= observed OD, corrected for unreacted menadione).

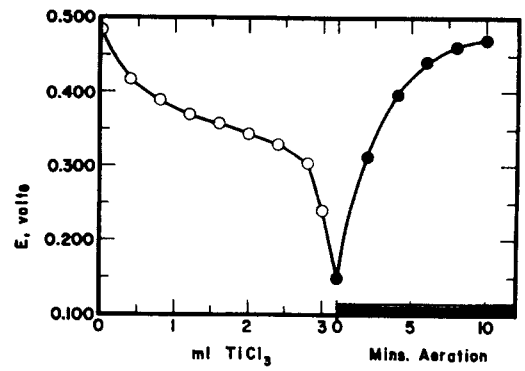


FIG. 6.—Changes in potential of platinum electrode (*vs.* normal hydrogen electrode) observed during reduction of thiodione by $TiCl_3$ at *p*H 1.0 (O), and during subsequent oxidation by air (●).

also does not occur, as was demonstrated by absence of any color change upon anaerobic addition of menadione of thiodione which had previously been reduced by $TiCl_3$.

Formation of Hydrogen Peroxide.—Since aerobic thioether formation had been shown to involve oxidation of reduced menadione, reaction mixtures were examined for presence of hydrogen peroxide. A positive test for peroxide was obtained by the method of Hart (1951), in which KI is converted to I_2. Since monosodium glutathione reacts with iodine, the quantitative determination of H_2O_2 was carried out in reaction mixtures containing a large excess of menadione. A 70% ethanol solution, initially containing 3×10^{-2} M menadione and 1.5×10^{-3} monosodium glutathione, was allowed to stand in air for 4 hours. At that time the solution was found to contain 1.21×10^{-3} M thiodione, and the concentration of H_2O_2, after correc-

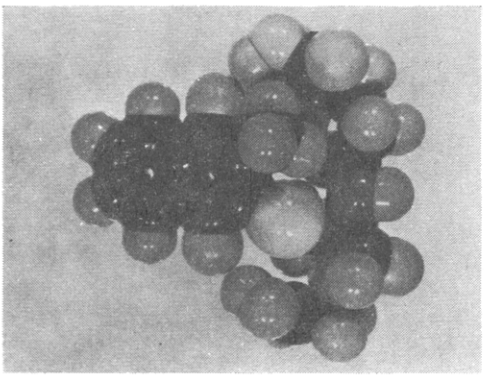


FIG. 7.—Molecular model of thiodione showing hydrogen bonding of glutamyl and glycyl carboxyls with quinone oxygens.

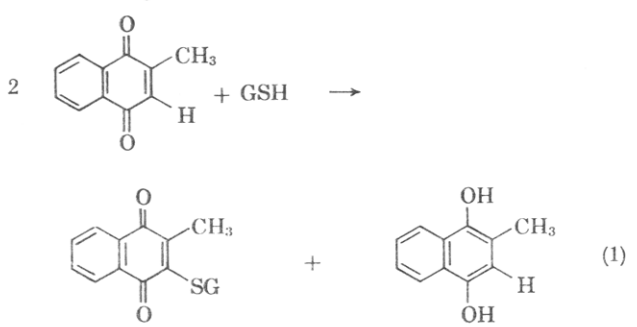
tion for the presence of 0.29×10^{-3} M unreacted monosodium glutathione, was 1.15×10^{-3} M. Thus, equimolar amounts of thiodione and hydrogen peroxide were formed during aerobic reaction.

DISCUSSION

Mechanism of Quinone-Thioether Formation.—Our experimental results show that (1) the yield of thiodione under anaerobic conditions is only half as great as that obtained in the presence of air; (2) a small rise in optical density, corresponding to oxidation of reduced menadione (rather than of reduced thiodione) occurs upon aeration of an air-free reaction mixture; (3) reduced menadione does not undergo thioether formation with monosodium glutathione; (4) thiodione does not oxidize reduced menadione; and (5) menadione does not oxidize reduced thiodione. These observations demonstrate that hydrogen eliminated during thioether formation is taken up by a second molecule of menadione. At no time was reduced thiodione detected during the substitution reaction between approximately equimolar amounts of reactants. Reduced thiodione can appear in the presence of a large excess of monosodium glutathione, but then only *after* the formation of the oxidized thioether.

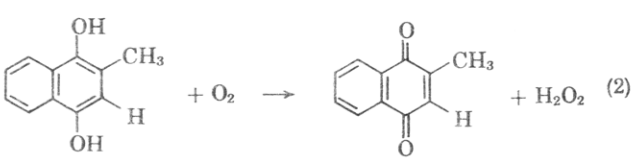
The reaction between menadione and glutathione (GSH) may thus be written as shown in equations (1) and (2).

Anaerobically:



(1)

On aeration:



(2)

Equation (1) shows that, anaerobically, no more than 50% of the total quinone can be converted to a thioether, and equation (2) shows that an equimolar amount of hydrogen peroxide is produced in air at the expense of the reduced intermediate.

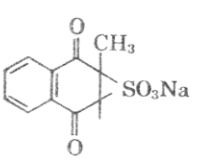
The evidence presented poses a problem regarding the mechanism of hydrogen migration during the substituted reaction. By analogy with the known charge-transfer complexing of benzoquinone with its dihydro-derivative in quinhydrone (Mulliken, 1952), it is possible that this migration occurs while a molecule of primary addition product and a molecule of menadione are held in close promixity. The relative abundance of menadione at the onset of the reaction, as well as possible steric hindrance in thiodione, favor complexing of thiodione with unsubstituted menadione rather than with a second molecule of thiodione.

The reaction mechanism described by equations (1) and (2) differs from the scheme postulated by previous workers (Snell and Weissberger, 1939; Schubert, 1947), according to whom the product of reaction between quinone and a thiol substance is a substituted hydroquinone. The procedure of Snell and Weissberger (1939) for addition of thioglycolic acid to *p*-benzoquinone was repeated. The alcoholic solution of benzoquinone developed an intense burgundy color (not mentioned by them) by the time half of an equimolar thioglycolic acid solution had been added. From equation (1), it will be recognized that an equimolar amount of thioglycolic acid constitutes a 100% excess for addition to *p*-benzoquinone. Thus, it is not surprising that a reduced quinone-thioether (a colorless solution) eventually results from addition of these quantities. Reduced benzoquinones, in contrast to reduced naphthoquinones, have such elevated redox potentials that they are stable to air and are not autoxidizable.

The procedure of Schubert (1947) for addition of thioglycolic acid to *p*-benzoquinone was also repeated. From the first drop of thioglycolic acid solution into the aqueous suspension of benzoquinone, an intense burgundy color was obvious. At no time did the "dark brown" color reported by Schubert appear. The burgundy color persisted, and no precipitate of reduced colorless product appeared until more than 0.8 mole of thioglycolic acid per mole of benzoquinone had been added. A colorless product (reduced substituted quinone) appeared only after an excess of thioglycolic acid had been added.

Structure of Thiodione.—The rapid disappearance of SH^- during formation of thiodione, together with the absence of oxidized glutathione, constitutes evidence that a carbon-sulfur bond is formed, the sulfur presumably attaching to a ring carbon. Reactions of glutathione with differently substituted naphthoquinones show that glutathione will add at positions 2 or 3, as has also been suggested by Fieser and Turner (1947), and by Fieser and Fieser (1956). This addition proceeds most rapidly at a chloro-substituted position and less rapidly at a hydrogen-substituted one; it does not occur at all at a methyl- or hydroxy-substituted one. The reaction therefore is seen to be proportional to the electron-withdrawing ability of the original substituent, indicating that glutathione reacts by nucleophilic substitution.

Carmack *et al.* (1950) have shown that a menadione-bisulfite addition compound can exist as 2-methyl-1,4-dioxotetralin-2-sulfonate:



Thiodione does not have an analogous structure, since

(1) thiodione is intensely colored, evidence of the presence of a quinone ring (the above bisulfite addition compound does not absorb above 385 mμ); and (2) the yield of thiodione was found to depend on the presence or absence of air, whereas formation of the above structure should be independent of the availability of oxygen.

Inspection of a molecular model of thiodione (Fig. 7) shows that hydrogen bonding can take place between the quinone oxygens and either the amino nitrogens or the carboxylic oxygens of the glutathione moiety and may thus stabilize the molecule. The redox potential of thiodione precludes involvement of the quinone oxygens in covalent bonding, resulting in ring closure, as was postulated by Kuhn and Beinert (1944) for the complex formed between benzoquinone and cysteine. The fact that reduced menadione, despite its somewhat lower redox potential, can exist in the presence of thiodione provides a further indication of stabilization of the quinone oxygens of thiodione by hydrogen bonding or steric hindrance.

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Studies on Quinone-Thioethers. II. Photochemical and Hydrolytic Cleavage of Thiodione*

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Anaerobic illumination with visible light of an aqueous solution of thiodione (2-methyl-3-glutathionyl-1,4-naphthoquinone) results in development of a burgundy color and a drop in potential. These effects result from the cleavage of thiodione with the formation of 2-methyl-3-mercapto-1,4-naphthoquinone (burgundy color) and dihydro-thiodione (lowered potential). In contrast, alkali-catalyzed cleavage of thiodione, in the dark, yields phthiocol and glutathione. The fact that phthiocol results from alkaline hydrolysis of thiodione (a quinone-thioether), and also from saponification of the acetone-soluble fat of human tubercle bacilli, suggests that the natural precursor of phthiocol may be a 2-methyl-naphthoquinone bound *via* a thioether linkage.

In the course of a study of the properties of thiodione (2-methyl-3-glutathionyl-1,4-naphthoquinone; Nickerson *et al.*, 1963), the substance was observed to be photolabile, as evidenced by rapid formation of a burgundy color in aqueous solutions exposed to visible light. Photochemical activity of quinone-thioethers has not been reported previously; however, inactivation and decomposition of the related vitamin K_1 by light (Almquist, 1936, 1937; MacCorquodale *et al.*, 1939; Ewing *et al.*, 1943) is well known. Therefore, photodecomposition of thiodione was studied, and a degradation product of the quinone moiety was identified.

Experimental

Materials.—The synthesis of thiodione has been described in the first paper of this series (Nickerson *et al.*, 1963). All substances used as chromatographic reference standards were tested and recrystallized if necessary until chromatographically pure. Nitrogen, from the Matheson Co., of 99.99% purity, was used to flush solutions to make them air-free.

The burgundy photodecomposition product of thiodione, later identified as 2-methyl-3-mercapto-1,4-naphthoquinone (prepared according to Nickerson, unpublished, and referred to as mercapto-menadione), was isolated from an illuminated thiodione solution as follows: 10 mg of thiodione in 50 ml water was illuminated by a 375-watt photoflood lamp at a distance of 10 cm. Nitrogen was flushed through the solution, which was kept cool under running water. At the end of 4 hours' illumination, the solution, now deep red and turbid, was acidified with HCl, whereupon it became pale yellow. It was exhaustively extracted with chloroform. When a sample of the pale yellow chloroform extract was shaken with aqueous NaOH, the characteristic burgundy color of illuminated thiodione appeared in the aqueous phase. The bulk of the chloroform extract was evaporated to dryness and yielded 2 mg of a dark reddish-brown product.

Potential Measurements.—For simultaneous measurement of potential and optical density during anaerobic illumination of thiodione solutions, an evacuable cell fitted with platinum and calomel electrodes (Strauss and

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