Oxidation of Biological Thiols:Glutathione-Indophenol Interactions*

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The rate, stoichiometry, and products for the oxidation of reduced glutathione (GSH) by 2,6-dichloroindophenol are dependent in part on the hydrogen-ion concentration and the presence of metal ions. In the absence of added metal ions, the major products of the reaction are the mono- and disubstituted *S*-glutathionyl-2,6-dichloroindophenols. These new dye derivatives were separated by chromatographic techniques and characterized by utilization of ^{14}C-labeled GSH. The maximum rate of formation of these substituted dye derivatives was observed in the *p*H range 6–7. Maximal yields of these derivatives were also obtained in this *p*H range. In this process GSH and indophenol react in the molar ratio 1:1. In acid and alkaline *p*H zones there is some suppression of the substitution reaction and a concomitant increase in the formation of oxidized glutathione (GSSG) as an end product. However under these conditions GSSG has not been obtained as a major product. Kinetic studies over a broad *p*H range indicate a common rate-limiting step resulting in the initial formation of an intermediate, GS:dye through nucleophilic attack of the GS^- anion on the indophenol component, effecting partial transfer of an electron. The mechanism of the competing pathways leading to the formation of substituted indophenols and GSSG is considered briefly. Added Cu^{2+} ions suppress the nucleophilic behavior of the thiol anion with formation of a thiol-copper complex which transfers electrons directly to the dye. In this case the stoichiometry is 2 moles GSH oxidized per mole dye reduced and, with sufficient Cu^{2+}, GSSG is formed in theoretical yield, as revealed by specific enzymatic assay with the enzyme glutathione reductase.

Investigation of the mechanism of oxidation of biologically important thiol compounds has presented a difficult experimental problem, and relatively little information is available for the *p*H zone characteristic of physiological processes.

Reactions of thiols with iodine, porphyrexides, and reversibly oxidizable dyes such as indophenols occur with stoichiometries that vary with *p*H, concentration of reactants, and the presence of heavy metal ions. For example, cysteine is oxidized at *p*H 4.5 by iodine with the stoichiometry 2 moles thiol per 1 mole iodine. However, at *p*H 7 the ratio may become 1:1, a sulfenyl iodide of the type R—S—I being formed (Fraenkel-Conrat, 1955; Cunningham and Neunke, 1959).[1,2] In contrast, oxidation of cysteine at *p*H 7 by the obligate 2-electron acceptor, iodosobenzoate ion, yields cystine; and GSH[3] is similarly oxidized. It was likewise observed in this laboratory[4] that the oxidation of glutathione by the stabilized radical (RAD) spirocyclohexylporphyrexide, a compulsory 1-electron acceptor, apparently yields an *S*-porphyrexide-substituted thiol (type, GS:RAD), again reducible with regeneration of the thiol. Finally, the ions of copper, iron, nickel, and other metals are thought (Barron, 1951) to exhibit more or less specific actions with respect to the course and rate of thiol oxidations.

The indophenol dyes have been used often as electron acceptors in dehydrogenase reactions and in various assay systems for ascorbic acid and thiols. Their interaction with glutathione and protein sulfhydryl groups may involve apparent inconsistencies. For example, 2,6-dichloroindophenol (DCI) has been observed to oxidize cysteine or GSH at approximately *p*H 4.5 with formation of a leuco product in a stoichiometry suggestive of the following reaction:

$$2GSH + DCI \longrightarrow G—S—S—G + DCI(H)_2 \quad (1)$$

However, Basford and Huennekens (1955) and others have studied the oxidation of various thiols by DCI at *p*H 7. They have observed rapid reduction of the dye to a leuco product in a process involving a molar ratio of indophenol and thiol of 1:1. This fact was at that time interpreted in accordance with the formalism (2):

$$RSH + DCI + H^+ \longrightarrow RS^+ + DCI(H)_2 \quad (2)$$

with the implication that 2 electrons had been removed from the sulfhydryl group resulting in its oxidation to the sulfenic acid stage (RS^+ or RSOH). In the presence of certain metal ions, including Cu^{2+}, the stoichiometry was found to be altered to the molar ratio, 2 moles RSH per mole DCI reduced. With some thiols such as thiophenol this ratio was obtained in the absence of metal.

Inasmuch as there is no conclusive evidence for the sulfenic acid mechanism, and because of theoretical objections to this postulation, we undertook an investigation of the mechanism of this reaction. Such a study also promised to offer leads with respect to the more general behavior of certain thiols under oxidizing conditions. On the basis of such observations as were discussed in the opening paragraphs, we postulated that an initial step in a one-to-one interaction of an indo-

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[1] L. Hellerman and P. J. Geiger (unpublished work), working with the enzyme glutamic dehydrogenase, observed the reversible reduction of such an intermediate by the prompt application of GSH or ascorbic acid.

[2] This is remindful of Raschig's (1915) suggestion of a somewhat related intermediate in the oxidation of thiosulfate by iodine ($^-O_3S\text{-}SH + I_2 \rightleftharpoons {}^-O_3S\text{-}SI + I^-$).

[3] Abbreviations: DCI, 2,6-dichloroindophenol; GSH, reduced glutathione; GSSG, oxidized glutathione; EDTA, ethylenediaminetetraacetate.

[4] L. Hellerman, K. A. Schellenberg, and P. J. Geiger, unpublished work. For a discussion of the electron acceptors, cf. Schellenberg and Hellerman (1959).

phenol (Ip) and GSH might consist in a one-electron transfer resulting in formation of an intermediate.

$$GS^- + Ip \longrightarrow (GS{:}Ip)^-$$

Subsequent events, especially transfer of GS^- for addition to a potentially quinoidal dye function to form a new GS-substituted reduced product oxidizable to the corresponding indophenol, were revealed in this investigation by means of kinetic, manometric, and especially chromatographic procedures. With excess GSH, the process may be forced to the ring addition of several GS-entities.

In contrast, the course of reaction is completely altered, even in a *p*H range (6.5–7) most favorable to these processes, when cupric ions are added to the reaction mixtures. Here the stoichiometry reverts to the more familiar oxidation of GSH to G—S—S—G, the quantitative nature of the action being demonstrable by specific enzymatic estimation of G—S—S—G produced. Recently, Hadler *et al.* (1963) observed a modification in the dye solubility and spectra after reduction by cysteine, suggesting an alteration in the DCI molecule which implied addition of the cysteine to a potentially quinonic ring of the dye.

Experimental

Reagents.—Reduced glutathione (Schwarz) and cysteine hydrochloride (Eastman) were assayed with *o*-iodosobenzoic acid (Hellerman *et al.*, 1941). Sodium salts of 2,6-dichloroindophenol, 2,6-dibromoindophenol, and phenolindophenol (Eastman) were assayed with ascorbic acid. All reagents were characterized by suitable methods and include L-ascorbic acid, glutathione-^{14}C reduced, 2.15 μc/mg (Schwarz), oxidized glutathione (Schwarz), glutathione reductase, 200 eu/mg (Calbiochem), and reduced TPN (Sigma). Deionized glass-redistilled water was used.

Kinetic Studies.—A typical assay system of 3 ml was made up with 1.0 ml of 0.1 M potassium phosphate buffer, *p*H 7 (33 mM), 0.15 ml 1 mM indophenol (50 μM), 0.10 ml mM thiol (33.3 μM), and 1.75 ml water. Final concentrations are indicated between the parentheses. Water, buffer, and indophenol were mixed in a 1-cm cuvet. The reaction was initiated by the addition of the electron donor. Absorbancy at 600 mμ was determined at 30-second intervals.

Sodium acetate buffer was used in the *p*H range 4.0–5.5, and the absorbancy was determined at 530 mμ. The effect of metal ions was determined at the final metal concentration, 3.3 μM. EDTA was present where noted at 3.3 mM.

The initial velocity, v_0, is defined as μmole indophenol reduced ml^{-1} min^{-1} $\times$ 10^2, and was calculated from the change in absorbancy over the first 30 seconds.

Identification and Separation of Reaction Products

A 4.0-ml reaction mixture contained 2.0 ml 5 mM thiol solution (2.5 mM) buffered with 0.1 M phosphate, *p*H 7, and 2.0 ml 5 mM indophenol (2.5 mM) in water. Final concentrations are indicated between parentheses. The reaction proceeded for 30 minutes at 23° and the products were reoxidized by passing oxygen gas through the mixture for 1 hour. The reactions were conducted at various hydrogen-ion concentrations as noted, but treatment with oxygen was always performed at *p*H 7.

Paper Chromatography.—The reoxidized reaction mixture was chromatographed on paper. The use of Whatman No. 1 paper gave excellent separation of the reaction products formed in the GSH-dichloroindophenol reaction. For separation of the reaction products from the cysteine-dichloroindophenol reaction, DEAE paper (Whatman DE20) was used. A total of 0.2 ml was spotted in 10-μl increments. The spot was allowed to dry after each application. The descending chromatographic technique was used with a solvent system 60% acetone–40% water (v/v). The solvent front moved approximately 30 cm after 8 hours at 3°. The chromatograph was dried and the reaction products at the visible spots were eluted with 3.0 ml of 0.5 M phosphate buffer, *p*H 7.

Column Chromatography.—50.0 ml of a cellulose calcium phosphate gel slurry containing 66 mg of powdered cellulose (Whatman) per ml and 10 mg calcium phosphate per ml were introduced into a 50-ml buret, resulting in a packed column 20 mm in length (Massey, 1960). The reoxidized reaction mixture (2.0 ml) was placed on the column. The column was eluted with acetone which was gradient-diluted with water. After collection of 4 ml per tube in a series of 30 tubes, the eluent was changed to water and an additional 15 such partitions were collected. The column was then cleared with 0.1 M phosphate buffer of *p*H 7.

GSSG Assay.—Residual indophenol in 2.35 ml of the reoxidized mixture resulting from reaction of GSH and dichloroindophenol was reduced by the addition of 0.1 ml of 0.01 M ascorbate and the following additions were made: 0.1 ml of 0.1 M EDTA, 0.05 ml of 3 mM TPNH, 0.1 ml glutathione reductase (20 eu/ml), and 0.30 ml H_2O. The reaction was initiated by the addition of enzyme. The change in absorbancy at 340 mμ $\times$ 0.206 is equivalent to the μmoles per ml of GSSG in the reaction mixture.

Results

Kinetics and Stoichiometry.—Some earlier titrimetric observations of Basford and Huennekens (1955) on the oxidation of glutathione and cysteine by dichloroindophenol were duplicated and confirmed in this laboratory with 2,6-dibromoindophenol. The kinetics, stoichiometry, and effects of metal ions in the reaction of the dibromoindophenol with various reducing agents at *p*H 7 are summarized in Table I.

Basford and Huennekens observed an increase in the velocity of the reaction with increasing hydrogen-ion concentration for the *p*H range 6.5–8.5. Tarbell (1961) has inferred from their data the rate equation

$$-\frac{d(RSH)}{dt} = k(H^+)(RSH)(DCI)$$

where RSH = cysteine and DCI = dichloroindophenol. Our observation of the reaction over a wider *p*H range indicates that the effect of hydrogen-ion concentration is more complex than the above expression indicates.

Table I
Reduction of 2,6-Dibromoindophenol with Various Reactants at *p*H 7[a]

Reactant	v_0	Stoichiometry
Ascorbic acid	6.1	1.00
GSH	1.3	1.15
GSSG	0	0
Cysteine	3.2	1.07
Cysteine; Cu^{2+}	3.8	1.96
Cysteine; Cu^{2+}; EDTA	3.0	0.71
Cysteine; Fe^{2+}	3.6	1.22

[a] Spectrophotometric assay at 600 mμ. Dibromoindophenol 50 μM; reactant 33 μM; phosphate buffer, *p*H 7, 33 μM; metal ion 3.3 mM; EDTA, 3.3 mM; v_0, initial velocity, μmoles dibromoindophenol reduced per ml per minute $\times$ 10^2 at 23°; stoichiometry, moles of reactant per mole of dibromoindophenol reduced.

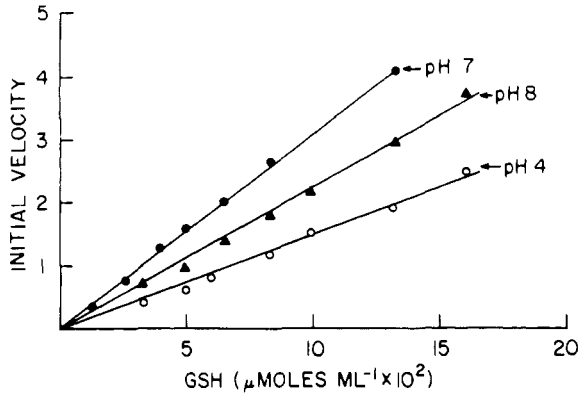


FIG. 1.—Effect of *p*H on the kinetics of the interreaction of GSH and dichloroindophenol. Each value represents the determination of initial velocity in a separate individual reaction system containing 0.05 μmole/ml dichloroindophenol, 33 mM phosphate buffer, and 33 mM acetate buffer adjusted to the *p*H noted. The GSH concentration was varied from 0.01 to 0.16 μmole/ml. The initial velocity represents μmoles of dichloroindophenol reduced per ml per minute × 100 and is calculated from the change in absorbance at 600 mμ for *p*H 7 and 8 and at 530 mμ for *p*H 4, over a 30-second period. The reactions were conducted at 23° in a Beckman DU spectrophotometer with a rate-recording chart.

Data presented in Table II indicate a *p*H optimum of 6.5 for the velocity of the reaction between glutathione and also cysteine with dichloroindophenol (cf. Hadler *et al.*, 1963). These data were collected with systems under anaerobic conditions to prevent the autoxidation of the dye. Reoxidation becomes a significant factor in the alkaline range.

The effect of alteration of the hydrogen-ion concentration upon the apparent stoichiometry of the GSH-indophenol reaction is in an opposite direction to that observed for the velocity. A 1:1 molar ratio is approximated in the *p*H range 5.5–6.5; thus as the velocity approaches a maximum the ratio approaches a minimum. When cysteine is involved the latter ratio drifts slowly with time below *p*H 5.5; a clear end point is not obtained.

The indophenol controls were assayed with ascorbic acid at each *p*H value and it was found that the acid instability of the dye was not a major factor for the time period over which the reaction was observed. Oxidation of ascorbic acid with dichloroindophenol did not exhibit a *p*H optimum. The velocity of the reaction increased with hydrogen-ion concentration and became too rapid to evaluate below *p*H 6.0.

TABLE II
EFFECT OF *p*H ON THE REACTION VELOCITY AND STOICHIOMETRY IN THE REDUCTION OF DICHLOROINDOPHENOL BY GLUTATHIONE OR CYSTEINE[a]

	Glutathione		Cysteine	
*p*H	v_0	Stoichiometry	v_0	Stoichiometry
8.0	0.98	1.60	2.35	2.30
7.0	1.12	1.25	2.80	1.25
6.5	1.75	1.15	3.08	1.04
5.5	1.67	1.12	2.55	1.03
5.0	1.17	1.43	2.10	
4.5	0.69	1.58	1.80	

[a] Spectrophotometric assay at 600 mμ. Dichloroindophenol, 50 μM; thiol, 33 μM; acetate or phosphate buffer, 33 mM; v_0, initial velocity, μmoles indophenol reduced per ml per minute × 10^2 at 23°; apparent stoichiometry, moles thiol per mole of dichloroindophenol reduced.

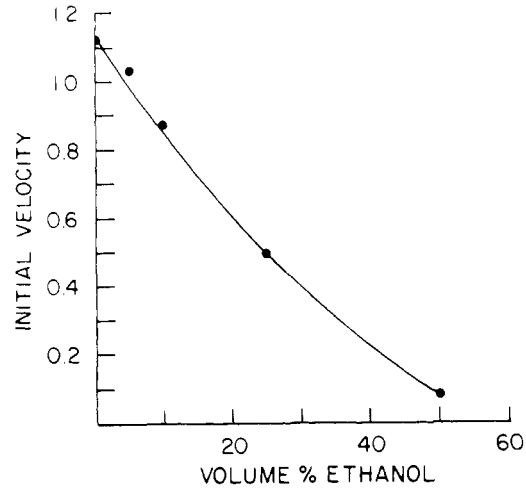


FIG. 2.—Effect of ethanol on the reaction velocity at *p*H 7. Spectrophotometric assays were conducted at 600 mμ on individual reaction systems containing dichloroindophenol, 50 μM; GSH, 33 μM; phosphate buffer, *p*H 7, 33 mM; and increasing volume % of ethanol. The initial velocity is μmoles of indophenol reduced per ml per minute × 100 at 23°.

While the stoichiometry and initial velocity vary with *p*H, kinetics remains first order with respect to the glutathione concentration over the *p*H range 4–8 (Fig. 1).

Effect of Ethanol.—In order to gage effects of solvent polarity we studied briefly the reaction rate in the presence of ethanol. A marked decrease in the initial reaction velocity was observed as the volume per cent ethanol in the reaction mixture was increased from zero to 50% (Fig. 2), and this was not accompanied by a change in the reaction stoichiometry.

Net Electron Transfer.—Net electron transfer for the overall reaction was evaluated by the use of reduced dye itself as an autoxidizable electron acceptor. Standard Warburg manometric technique was used. Main compartment additions were 1.35 ml of 0.5 M phosphate buffer, *p*H 7; 1.00 ml 5 mM dichloroindophenol; 0.10 ml catalase (5000 units). The sidearm addition was 0.75 ml of 5 mM thiol reagent or ascorbic acid. For the controls the main compartment contained the same reagents and the sidearm contained 0.75 ml of water. The flasks were equilibrated with air at 30°. The system was closed and the reaction was initiated by tipping the reductant from the sidearm into the main compartment. The oxygen uptake was recorded at intervals for a 3-hour period, complete stability being approached for all flasks. The calculation of the oxygen consumed permits determination of the net electron exchange for the reaction.

A reaction type implying oxidation of RSH to the sulfenic acid stage would involve a net 2-electron transfer per mole of thiol, whereas oxidation to the disulfide would provide 1 electron per mole of thiol

TABLE III
OXYGEN CONSUMPTION AND NET ELECTRON TRANSFER

Electron Donor[a]	Gram Atoms Oxygen Consumed/Mole Electron Donor
GSH	0.80
Cysteine	0.90
Ascorbic acid	0.96

[a] Indophenol present was in 25% molar excess with respect to electron donor.

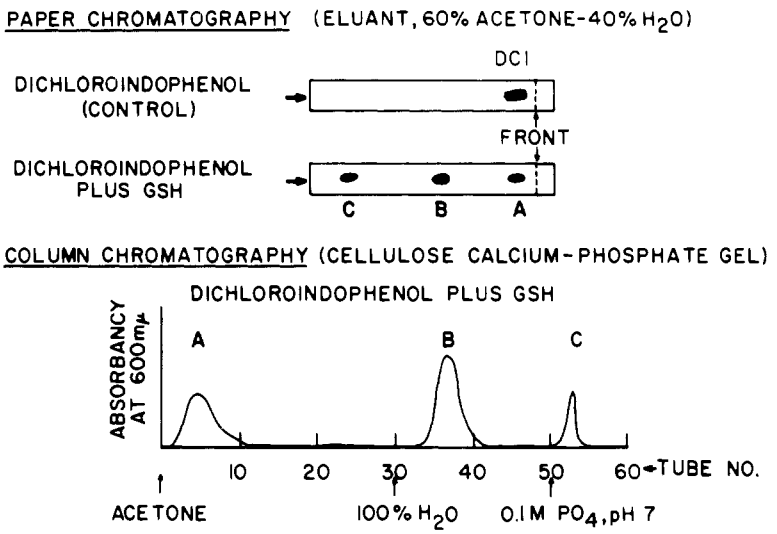
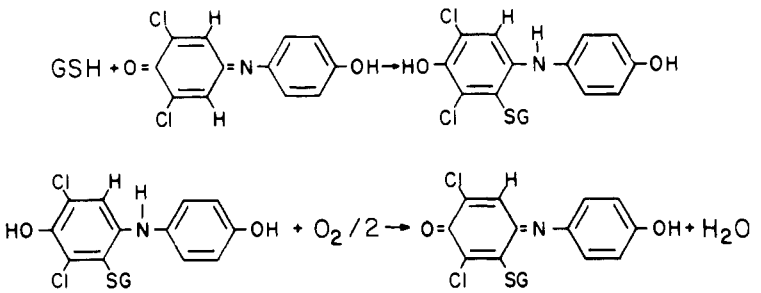


FIG. 3.—Chromatographic identification of GSH-dichloroindophenol reaction products (schematic). A reaction mixture containing 2.5 mM dichloroindophenol, 2.5 mM GSH, and 0.1 M phosphate buffer, *p*H 7, was allowed to react for 30 minutes at 23°. The leuco dyes were reoxidized with oxygen and samples of the reaction systems were chromatographed on paper and cellulose-calcium phosphate gel at 0° (see Experimental).

oxidized. Both these reactions would result in complete recovery of the initial indophenol dye after reoxidation of the reaction mixture.

On the other hand, net electron transfer for a thiol addition to a quinoid ring of the dye would involve an apparent 2-electron transfer, but reoxidation of this reaction mixture would yield, in addition to excess indophenol used, a new glutathionyl-substituted indophenol, represented by the equation in Scheme 1.



SCHEME I

Results are recorded in Table III. It will be seen that for both cysteine and reduced glutathione, 1 g-atom of oxygen (corresponding to a 2-electron transfer) was consumed per mole RSH. This rules out oxidation of RSH to the disulfide under these conditions; and the presence of sulfur, in the new GS-indophenol product (made evident by chromatographic examination of the unlabeled and ^{14}C-labeled products as described below) permitted the conclusion that a glutathionyl-substituted product, rather than a sulfenate, had been formed almost quantitatively.

Separation of Reaction Products.—Paper chromatographic identification of the reaction products was achieved with the use of a solvent system 60% acetone–40% water (v/v) and Whatman No. 1 paper as outlined in the experimental section. This permitted excellent separation for the products of the glutathione-indophenol process but was unsatisfactory for the cysteine system. With DEAE-paper the cysteine products were resolved. The following studies were performed predominantly with the glutathione system because of the sharp resolution obtainable and in view also of the difficulty in quantitatively eluting the cysteine products from the DEAE-paper.

The glutathione-DCI products were resolved as three distinct spots (Fig. 3). For reference these spots are coded A, B, and C. The control DCI solution gave one spot, coded DCI. As anticipated, GSSG in place of GSH did not give products B and C; furthermore, GSH treated with iodoacetamide, to alkylate the sulfhydryl group, failed to produce such products. The cysteine-DCI system gave four spots on DEAE-paper chromatography, coded a, b, c, d (Table IV).

The close correlation between spot DCI, the control dye, and the spot A of the glutathione system suggests their identity; this will be confirmed presently. Spot B will be identified as an addition product of 1 molecule of GSH with the dye, DCI-(SG); Spot C will be identified as the addition of 2 molecules of GSH with the dye, DCI-$(SG)_2$.

Compounds A, B, and C were separated by column chromatography on a cellulose–calcium phosphate gel. The collecting tubes were assayed at 600 mμ (Fig. 3).

Characterization of the GSH-Dichloroindophenol Reaction Products.—The GSH-dichloroindophenol reaction mixture was reoxidized at *p*H 7 and the reaction products were separated by column chromatography into products A, B, and C. Comparison of these three products with the original dichloroindophenol dye indicates correspondence of compound A and dichloroindophenol with respect to spectral properties as well as pK' and R_F values; compounds B and C are new dye derivatives. The comparative data are summarized in Table V.

^{14}C-labeled reduced glutathione was used to determine whether glutathione was incorporated into the

TABLE IV
PAPER CHROMATOGRAPHY OF REACTION PRODUCTS[a]

Reaction System	Paper	Spot	Color	R_F	Total Dye (%)
DCI (control)	Whatman #1	DCI	Purple	0.93	100
GSH; DCI	Whatman #1	A	Purple	0.93	28.0
		B	Blue-purple	0.65	59.8
		C	Blue-green	0.16	12.2
DCI (Control)	DEAE paper	DCI′	Blue	0.50	
Cysteine; DCI	DEAE paper	*a*	Blue	0.50	
		b	Green (weak)	0.34	
		c	Brown to blue	0.08	
		d	Dark brown	0.00	

[a] A reaction system containing 2.5 mM thiol, 2.5 mM dichloroindophenol, and 0.1 M phosphate, *p*H 7, was reoxidized following a 30-minute reaction period at 25°. Samples were paper chromatographed and the visible spots were eluted and assayed at 600 mμ (see Experimental). Molar absorption coefficients reported in Table V were used to calculate the percentage of the total dye appearing in each eluted spot.

TABLE V
COMPARISON OF SPECTRA, MOLAR ABSORPTION COEFFICIENTS, AND *p*K′ COEFFICIENTS OF THE GSH-DICHLOROINDOPHENOL REACTION PRODUCTS WITH CORRESPONDING VALUES FOR UNREACTED DICHLOROINDOPHENOL[a]

	Spectrum				
	*p*H 7		*p*H 3		
Chromatographed Product	λ_{max} (mμ)	Molar Absorption Coefficient[c]	λ_{max} (mμ)	$\frac{A_{390\ m\mu}}{A_{\lambda max}}$[d]	*pK′*[b]
Control DCI	612	20	510	0.41	5.82
GSH-DCI Reaction					
A	610	20	510	0.41	5.80
B	640	13	480	0.92	5.38
C	635	9	490	1.75	5.50

[a] Two ml of a reaction mixture containing 2.5 mM dichloroindophenol, 2.5 mM GSH, and 0.1 M phosphate buffer, *p*H 7, was reoxidized following a 30-minute reaction period at 23°. The reoxidized sample was chromatographed on a cellulose–calcium phosphate gel column (see Experimental and Fig. 3). Products A, B, and C were separated and compared to a similarly chromatographed dichloroindophenol control. [b] *pK′* determined as the *p*H at which there is a 50% reduction in the absorbance at λ_{max}, 23°. [c] $M^{-1}\ cm^{-1} \times 10^3$ at 600 mμ; determined by assaying the dye product with ascorbic acid and assuming a 1:1 molar ratio. [d] Ratio of the absorbancy at 390 mμ to the absorbancy at λ_{max}, at *p*H 3.

TABLE VI
INCORPORATION OF LABELED GSH-^{14}C INTO REACTION PRODUCTS AT *p*H 7[a]

Chromatogram	Visible Spots (R_F)	Labeled Spots (R_F)	Distribution of Total Label (%)	Moles GSH Incorporated/ Mole of Product
1. GSH-^{14}C and DCI				
A	0.83		0	0
B	0.65	0.65	33.6	0.88
C	0.23	0.23	56.3	1.87
Other	None	Diffuse	10.1	
2. GSH-^{14}C alone		0.48	100.0	
3. GSSG-^{14}C alone		0.27	100.0	

[a] A reaction system containing 2.5 mM dichloroindophenol, 2.5 mM GSH with a specific activity of 4.52 μc per mmole, and 0.1 M phosphate buffer, *p*H 7, was allowed to react for 30 minutes at 23° and was then reoxidized and chromatographed on paper (see Experimental). The chromatogram was dried and radioactivity was counted in a strip counter (Scanogram II, Atomic Accessories Inc.). The labeled spots were removed and counted in a Geiger-Müller counter. The visible spots were eluted and assayed at 600 mμ and the incorporation of labeled GSH in the products A, B, and C, was determined. These values were compared to GSH-^{14}C and GSSG-^{14}C controls.

modified dye products. Of the label, 89.9% appeared in compounds B and C, while compound A was devoid of the label. The activity in compound B was compatible with the structure DCI-(SG) and compound C was indicated to be DCI-$(SG)_2$ (Table VI).

Factors Affecting the Formation of the Substituted Reaction Products (B and C)

Concentration of Reactants.—The proportion of A, B, and C recovered from the DCI-GSH process is dependent upon the initial molecular ratio of GSH and dichloroindophenol. With a fixed initial concentration of dichloroindophenol and varying amounts of GSH, reactions were conducted under nitrogen for 30 minutes after which sufficient oxygen was introduced for reoxidation of the indophenols. Samples were then paper-chromatographed and the visible spots corresponding to products A, B, and C were eluted and assayed at 600 mμ. The data in Table VII illustrate the increase in product C at the expense of A and B as the ratio GSH:dichloroindophenol is increased. At very high ratios the major portion of the dye was converted to product C with relatively negligible recovery of products A and B (Table VII).

Effects of *p*H.—When the reaction of GSH and dichloroindophenol had been conducted at various *p*H levels a maximum reaction rate was observed at *p*H 6.5. At this same *p*H level there was observed

TABLE VII
EFFECT OF RELATIVE INITIAL CONCENTRATIONS OF REACTANTS ON THE FORMATION OF SUBSTITUTED REACTION PRODUCTS B AND C[a]

Molar Ratio GSH/DCI	Percentage of Total Dye Appearing as Product		
	A	B	C
0.25	74.1	21.8	4.1
0.50	61.0	33.5	5.4
1.00	27.2	58.6	14.2
1.50	10.9	35.1	55.0
2.00	1.5	22.0	76.5

[a] Varying ratios of GSH to dichloroindophenol concentration were obtained in individual reaction vessels by maintaining the dichloroindophenol concentration constant at 2.5 mM and varying the GSH concentration over the range 0.62–5 mM. The reactions were conducted at 23° under atmospheric conditions in 0.1 M phosphate buffer, *p*H 7. After a reaction time of 30 minutes the leuco dyes were reoxidized with oxygen. Samples from each vessel were paper-chromatographed (see Experimental) and spots A, B, and C were eluted and assayed at 600 mμ.

also the minimum molar ratio for the interaction of dye and GSH, viz., 1:1 (Table II).

Accordingly, the effect of *p*H on the relative formation of substituted reaction products B and C was determined. Individual reaction vessels containing equal molar concentrations of dichloroindophenol and GSH were observed at various *p*H levels; after 1 hour the *p*H of all systems was adjusted to 7 and the leuco indophenols were reoxidized with oxygen. Samples of each system were paper-chromatographed and the visible spots A, B, and C were eluted and assayed at 600 mμ. The *p*H optimum for the formation of the substituted reaction products B and C was found to be approximately 6 (Fig. 4). From these results and others treated in the foregoing it is evident that in the *p*H region 6–6.5 there is optimal formation of glutathionyl-substituted products associated with a maximal reaction velocity. At this same *p*H level there is minimal interaction of GSH and indophenol in the molar ratio 2–1, with formation of GSSG.[5]

Effect of Cu^{2+}.—All studies conducted with added Cu^{2+} ions were performed under anaerobic conditions to prevent autoxidation of the thiol (GSH or cysteine). Deionized glass-distilled water was used, and buffer salts (reagent grade) were twice recrystallized. The marked effect observed in the presence of added cupric ion with respect to the stoichiometry of the action of dichloroindophenol with either cysteine or reduced glutathione is well illustrated by the data of Figure 5. Increasing the concentration of Cu^{2+} in the reaction system progressively alters the molar ratio of (GSH:DCI) to a final ratio (2 GSH:DCI) denoting oxidation of 2 moles GSH/mole DCI reduced. The quantity of Cu^{2+} required for *complete transition* to the 2:1 ratio is approximately 0.5–0.75 of the thiol concentration. The effects of added Cu^{2+} are reflected in the character and yield of the products formed. The formation of oxidized glutathione (GSSG) was followed by the glutathione reductase assay procedure described under Experimental. Increasing concentration of Cu^{2+} results in a correspondingly increased production of GSSG as a reaction product here of thiol oxidation

[5] From Fig. 4 it is evident that in the extremes of *p*H (4–9) and under the conditions used the substitution reactions are still a major pathway, accounting for most of the GSH decrease. Enzymatic estimation of the GSSG formed at these extremes discloses yields far below prediction on the basis of 2–1 stoichiometry. This aspect is under further investigation.

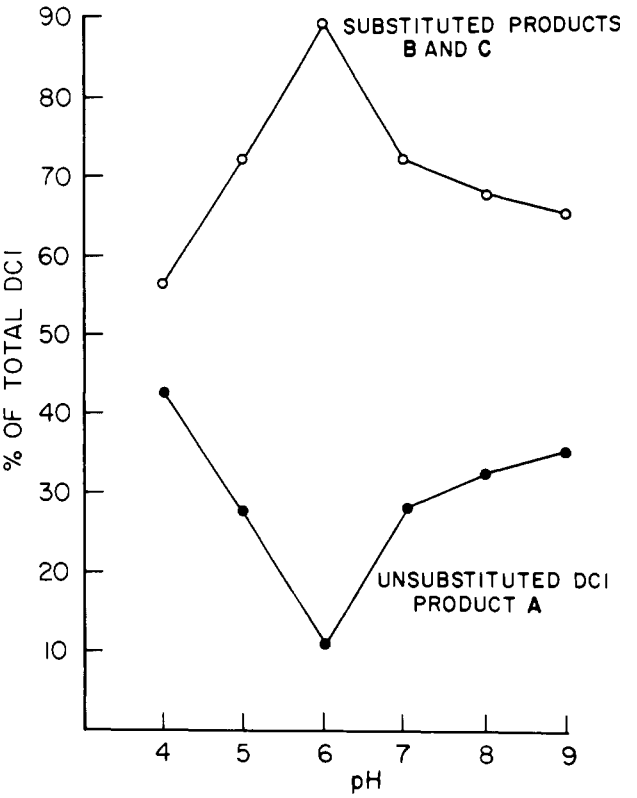


FIG. 4.—Effect of *p*H on the formation of the substituted reaction products B and C and the recovery of the unsubstituted product A. Reactions were conducted individually from *p*H 4 to 9 in systems containing 2.5 mM dichloroindophenol, 2.5 mM GSH, 50 mM phosphate buffer, and 50 mM acetate buffer. These reactions were conducted at 23° under anaerobic conditions for 1 hour. The *p*H was then adjusted to 7 in each reaction vessel and the leuco dyes were reoxidized with oxygen. Samples from each vessel were paper-chromatographed and the corresponding spots A, B, and C were eluted and assayed at 600 mμ (see Experimental).

(Fig. 6). The yield approaches the theoretical. Moreover, the presence of Cu^{2+} is observed to decrease the formation of the glutathionyl-substituted products B and C, resulting in a large increase in the recovery of the unsubstituted dye product, A. This is observed by allowing reoxidation followed by chromatographic separation on a cellulose–calcium phosphate column (see Table VIII). It is apparent, therefore, that the presence of Cu^{2+} markedly inhibits the formation of the glutathionyl-substituted dye products resulting in an increased formation of the disulfide GSSG.

Magnetic Susceptibility Studies.—Initial magnetic susceptibility studies at 23° with a Varian Gouy balance have indicated the rapid decay of a paramagnetic substance within a 10-minute period when the reaction between GSH and dichloroindophenol is conducted at *p*H 4 or 9.5. This paramagnetic decay was not observed at *p*H 7. These reactions were conducted at a 5 mM concentration of GSH and dichloroindophenol in the presence of 25 volume % ethanol.[6]

Related Dyes.—It was possible to identify analogous glutathionyl-substituted reaction products between GSH and dyes related to DCI including phenolindophenol, 2,6-dibromoindophenol, as well as phenol blue and Bindschedler's green. Preliminary studies revealed the same generality of the type of reaction that was observed with dichloroindophenol. Such studies will be described elsewhere.

[6] We wish to thank Mr. Byron D. McLees for aid in the magnetic susceptibility studies.

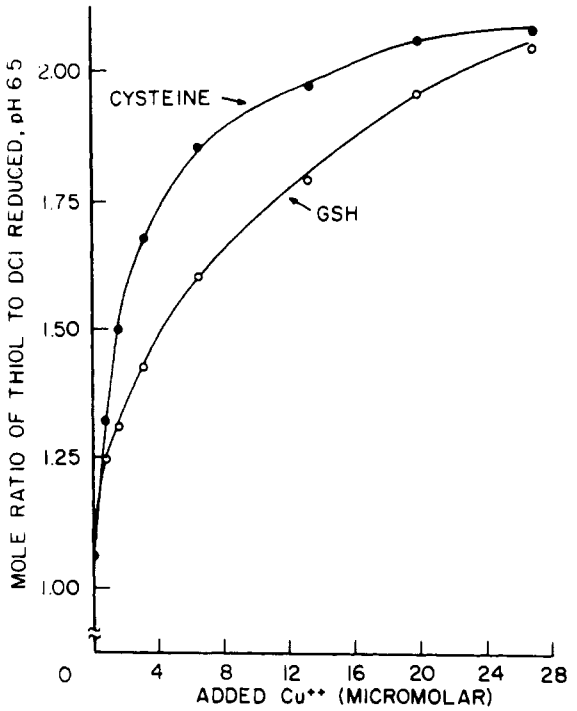


FIG. 5.—The effect of varying cupric ion concentration on the reaction stoichiometry between GSH or cysteine and dichloroindophenol. Separate reaction mixtures containing 50 μM dichloroindophenol, 33 μM thiol, and 33 mM phosphate buffer, *p*H 6.5, were allowed to react under anaerobic conditions at 23° in the presence of varying concentrations of cupric ions, final concentration in reaction mixtures from 0 to 27 μM. Each reaction system was allowed to reach completion and the stoichiometry is represented as the moles of GSH per mole of dichloroindophenol reduced as assayed by the change in absorbance at 600 mμ.

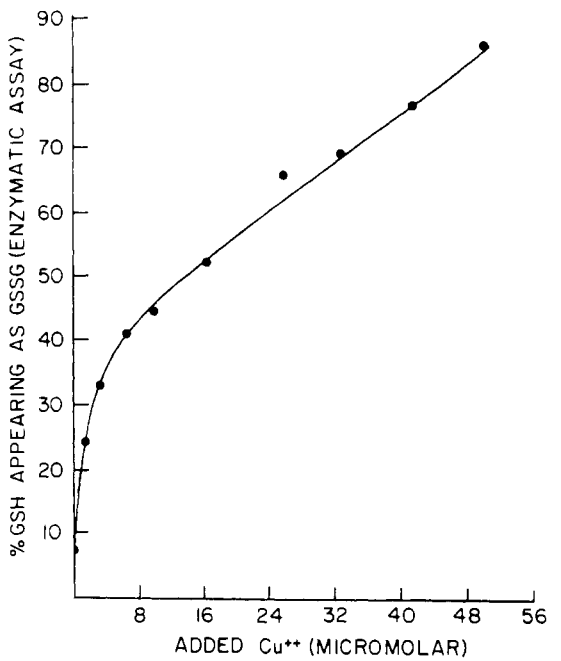


FIG. 6.—The effect of varying the cupric ion concentration Cu^{2+} on the formation of GSSG as the reaction product of the GSH-dichloroindophenol reaction. Each value represents a separate, individual reaction system with an added Cu^{2+} concentration in the range 0–52 μM, 150 μM dichloroindophenol, 100 μM GSH, and 0.1 M phosphate buffer, *p*H 6.5. The reactions were conducted under anaerobic conditions at 23° for 30 minutes following which each reaction vessel was assayed for GSSG with a specific enzymatic glutathione reductase assay procedure (see Experimental). The data are represented as the percentage of the total GSH in the reaction mixture which has been converted to GSSG.

TABLE VIII
EFFECT OF ADDED Cu^{2+} ON THE FORMATION OF THE SUBSTITUTED REACTION PRODUCTS B AND C[a]

Reaction System	Total Dye (%) A	B	C
GSH and DCI	42	32	26
GSH, DCI, and Cu^{2+}	81	18	0

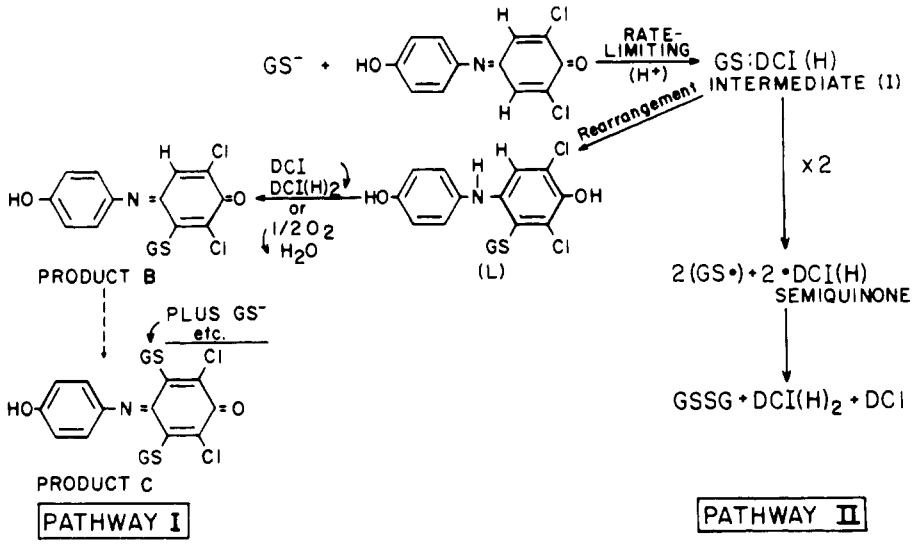
[a] 2.0 ml of a reaction mixture containing 2.5 mM dichloroindophenol, 2.5 mM GSH, and 0.1 M phosphate buffer, *p*H 7, was reoxidized following a 30-minute reaction period conducted at 23° under anaerobic conditions. This reoxidized sample was chromatographed on a cellulose–calcium phosphate gel column (see Experimental). Products A, B, and C were separated and assayed at 600 mμ. These values were compared with values from a similar system to which had been added as cupric chloride 0.25 mM Cu^{2+} prior to the addition of the GSH to the reaction mixture.

DISCUSSION

Quantitative chromatographic analysis indicates that the major product of the oxidation of glutathione by the indophenol dyes are the glutathionyl-substituted dye derivatives. This is particularly the case when the reaction is conducted in the middle *p*H range (*p*H 6–7) and in the absence of certain metal ions, e.g., Cu^{2+}. When GSH was oxidized by dichloroindophenol, two glutathionyl-substituted dye derivatives were identified (B and C). These new dye derivatives were separated and partially characterized. Labeling experiments clearly demonstrated that product B resulted from the incorporation of one residue of GSH per molecule of dye. Product C was a disubstituted derivative and contained two GSH residues per molecule. Increasing the ratio of GSH to indophenol in the reaction mixture provides a higher yield of the di-substituted product (C).

Introduction of thiol substituents into quinones, with formation of thioether linkages, has been known since the early work of Troeger and Eggert (1896) and of Bongartz (1888). A somewhat limited insight has been gained of the reaction mechanism involved (Schubert, 1947; Meguerian, 1955; Weissberger and James, 1939; Weissberger and Snell, 1939; Nickerson *et al.*, 1963). Schubert (1947) has shown that thiol groups can be added to all of the available positions of the benzoquinone ring.

In the absence of added metal ions the rate of the glutathione-dichloroindophenol reaction is strongly influenced by the hydrogen-ion concentration as well as the polarity of the solvent system. This suggests the involvement of a charged species in the rate-determining step. This initial reaction velocity is likewise dependent on the concentration of GSH and indophenol and is observed to be first order with respect to the GSH concentration over the broad *p*H range 4–8. This would indicate a common rate-limiting pathway. We postulate the initial formation of an intermediate, GS:(DCI), through nucleophilic action of the GS^- anion upon the protonated (uncharged) species of oxidized indophenol, DCI (not DCI^-), effecting a partial transfer of an electron from GS^- to the DCI component. It would be anticipated that the *p*H optimum for the reaction velocity would occur when the concentration product $[GS^-][DCI]$ is maximal. This would obtain at approximately *p*H 7, inasmuch as pK' for GSH is 9.2 and for oxidized indophenol 5.7. Experimentally, the value for maximal rate has been found in this work to be at 6.5.



SCHEME 2

As stated above, the major pathway for the overall process results in production of S-glutathionyl-2,6-dichloroindophenols. Thus, the fate of the presumed initial intermediate involves a "rearrangement process" to yield such derivatives. However another pathway from this intermediate exists. In the acid and alkaline range there is decrease in the formation of the substituted dyes with some concomitant increase in the formation of GSSG, according to the net overall process 2 GSH + DCI → GSSG + $DCI(H)_2$. The suppression of the substitution reaction and the approach to a 2:1 stoichiometry at high and low *p*H values possibly can be explained in part by the increased stability of the semiquinone form of the dye in these ranges. It was stated by Schwarzenbach and Michaelis (1938) that "the observation of all three dyestuffs, Bindschedler's green, phenol blue and phenol-indophenol, indicates the following general behavior: strong semiquinone formation in alkaline and acid range and comparatively small semiquinone formation in the middle *p*H ranges." This increased stability of the semiquinone could explain our observation of decay in reaction mixtures of a paramagnetic substance at *p*H 4.0 and 9.5 and its absence at *p*H 7. Other factors such as the increased stability of the reduced dye in the acid range and the enhanced reductive properties of GSH in the alkaline range also may aid significantly in elucidation of *p*H effects.

The pathways for the glutathione-indophenol process, in the absence of metal ions, are illustrated schematically in Scheme 2.[7] Pathway I, the predominant action, favored optimally at *p*H 6, involves rearrangement of the intermediate (I) to a leuco dye product (L). This is oxidized to form product B, which may be transformed by a similar series of events to give product C. The reaction stoichiometry for pathway I under anaerobic conditions is 1:1. Pathway II is favored in the more acid and alkaline ranges and might involve dissociation of the intermediate to the semiquinone and a transitory thiol radical. The disulfide would form rapidly and the semiquinone would dismute as indicated. Pathway II, particularly in dilute solutions, seemingly approaches a molar ratio of 2 moles of GSH required to reduce 1 mole of DCI. However, in the absence of added metal ions *GSSG at no time has been obtained as the major product* of GSH oxidation by DCI.

[7] cf. Hadler and Erwin, 1963.

Metal Effects.—The addition of cupric ions, which form complexes with thiol groups, apparently acts to prevent the formation of the initial intermediate, suppressing the formation of the substituted dye derivatives, *even at pH 6.5.* In the presence of metal ions the thiol groups of cysteine and glutathione may transfer electrons directly or indirectly to the dye, forming the disulfide GSSG by a mechanism differing from pathway II. With Cu^{2+} additions approaching the initial GSH concentration, *GSSG becomes the sole product* of oxidation by indophenol. Thus, while a catalytic element may be introduced at low Cu^{2+} concentrations, the data obtained in this investigation (Figs. 5 and 6) point to electron transfer directly from thiol-copper complexes to the dye electron acceptor. This implies, in effect, suppression of an oxidative process utilizing the marked nucleophilic characteristic of sulfhydryl groups. Thus the progressive addition of Cu^{2+} results efficiently in alteration in the pattern of oxidation of cysteine or GSH by indophenol in the molar ratio 1:1, in the direction 2 moles RSH to 1 mole indophenol reduced, and this likewise is reflected in the pattern of the reaction products. In sum, the yield of substituted dye products B and C is significantly diminished, and at the same time GSSG is formed in the theoretical yield, as revealed by specific enzymatic assay with the enzyme glutathione reductase.

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Preparation of Crystalline α-D-Galactosamine-1-Phosphoric Acid and Its Conversion to UDP-*N*-Acetylgalactosamine*

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A simple method has been developed for the enzymatic preparation of α-D-galactosamine-1-phosphoric acid in gram quantities, and the product has been crystallized. The compound, obtained in 60% yield, has an $[\alpha]_D^{25} = +142.6°$ ($c = 2.0\%$ in H_2O). After *N*-acetylation with acetic anhydride, the resulting *N*-acetyl-α-D-galactosamine-1-phosphate was crystallized as the potassium salt which had an $[\alpha]_D^{25} = +112.4°$ ($c = 2.9\%$ in H_2O). UDP-*N*-acetylgalactosamine was prepared by reacting mono-(tri-*n*-octylammonium)-*N*-acetylgalactosamine-1-phosphate with uridine-5′-phosphoromorpholidate in anhydrous pyridine. As the product, UDP-*N*-acetylgalactosamine, is synthesized by an unambiguous route from the α anomer of galactosamine-1-phosphate, it also must be the α anomer. A new procedure has been devised for the assay of galactokinase that is generally applicable to enzyme-catalyzed reactions where reducing sugars are converted to glycosides. This assay utilizes sodium borohydride for reduction of excess substrate to the corresponding sugar alcohol. The glycosidic bond, the aglycone being phosphate in the present case, is cleaved with acid, and the resulting sugar is measured by conventional techniques.

N-Acetyl-D-galactosamine (2-acetamido-2-deoxy-D-galactose) is found in many macromolecular substances (Kent and Whitehouse, 1955); a fungal polysaccharide (Distler and Roseman, 1960) is exceptional in that it contains D-galactosamine as well as the *N*-acetyl derivative. The precursor of the macromolecules is probably UDP-*N*-acetylgalactosamine, enzymatically derived from UDP-*N*-acetylglucosamine (Maley and Maley, 1959; Glaser, 1959). Although both enzymatic and chemical methods can be used for the synthesis of UDP-*N*-acetylgalactosamine (Davidson and Wheat, 1963; Kim and Davidson, 1963), these procedures are not convenient for preparing pure, specifically labeled material in substrate amounts.

Sugar-nucleotides similar to UDP-*N*-acetylgalactosamine can be synthesized by a general method (Roseman *et al.*, 1961), but only if the corresponding 1-phosphate derivative of sugar is readily available. The problem of obtaining galactosamine-1-phosphate in adequate amounts, specifically the α anomer, was therefore the primary purpose of this investigation. A simple method has been developed for the enzymatic synthesis of D-galactosamine-1-phosphate in gram quantities and, in addition, the pure compound has been crystallized. The hexosamine-phosphate was quantitatively *N*-acetylated by the usual procedure (Distler *et al.*, 1958), and the *N*-acetylgalactosamine-1-phosphate was converted to the desired UDP-*N*-acetylgalactosamine.

The original procedure for the enzymatic synthesis of galactosamine-1-phosphate (Cardini and Leloir, 1953) was repeated and gave only small amounts of material, a result explained by the observation that yeast galactokinase exhibits a high K_m for galactosamine (Alvardo, 1960). By modifying the original conditions and applying methods previously used for isolating the crystalline 6-phosphate esters of glucosamine, galactosamine, and mannosamine (Distler *et al.*, 1958; Jourdian and Roseman, 1962), we have obtained crystalline galactosamine-1-phosphate in better than 60% yield.

Since the enzyme incubations required high galactosamine concentrations, it was not practical to assay the crude reaction mixtures by the usual method, i.e., determining substrate disappearance. A new assay technique was therefore devised that made it possible to measure product formation. In this assay, the excess substrate is reduced with sodium borohydride, to which the product is resistant because it contains a protected carbonyl group. This principle is generally applicable to enzyme reactions where reducing sugars are converted to nonreducing products such as glycosides. The product is measured, in the case of galactosamine-1-P, by *N*-acetylation, hydrolysis, and a modified Morgan-Elson method. With galactose, the product is measured as reducing sugar after the borohydride treatment, hydrolysis, and deionization.

Experimental Procedure

Materials.—The yeast used in this study was a galactose-adapted strain of *Saccharomyces fragilis*, purchased from the Sigma Chemical Co. Galactosamine-hydrochloride was prepared as previously described (Roseman and Ludoweig, 1954). Phosphoenolpyruvate, 3-phosphoglyceric acid, UMP, and ATP were commercial materials. UMP-morpholidate was prepared as previously described (Moffatt and Khorana, 1961).

Methods.—The following substances were determined by the indicated procedures: protein by the

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