Haloacetol Phosphates. Potential Active-Site Reagents for Aldolase, Triose Phosphate Isomerase, and Glycerophosphate Dehydrogenase. I. Preparation and Properties*

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ABSTRACT: 3-Chloro-, 3-bromo-, and 3-iodoacetol phosphate, reactive derivatives of dihydroxyacetone phosphate, were synthesized as potential active-site reagents for aldolase, triose phosphate isomerase, and glycerophosphate dehydrogenase. The dimethyl ketals of the reagents were obtained as crystalline biscyclohexylammonium salts by a series of reactions starting with 3-halo-1,2-propanediols. At pH 10.2, haloacetol phosphates are rapidly converted into dihydroxyacetone phosphate, but at pH 6–8 are fairly stable. Sulfhydryl is the only functional group of free amino acids that reacts with haloacetol phosphates. The SH group of glutathione is covalently modified by chloro- and bromoacetol phosphate to form a presumed *S*-alkyl derivative. Iodoacetol phosphate oxidizes cysteine and glutathione predominantly to disulfides. These studies suggest that haloacetol phosphates will react preferentially with SH groups of proteins.

Incorporating into a protein reagent those structural features of a substrate necessary for its binding to a given enzyme gave active-site specific reagents (Schoellmann and Shaw, 1963; Shaw *et al.*, 1965; Khedouri *et al.*, 1966; Cuatrecasas *et al.*, 1969). This approach to the labeling and identification of active-site components, which Wofsy *et al.* (1962) called "affinity labeling," was applied successfully to a number of enzymes, especially proteases. A comprehensive monograph (Baker, 1967) and review article (Singer, 1967) concerning the theory and application of affinity labeling are available.

I have prepared the 3-chloro, 3-bromo, and 3-iodo derivatives of acetol phosphate (haloacetol phosphates), compounds structurally similar to DHAP,[1] as potential active-site specific reagents for aldolase, triose phosphate isomerase, and glycerophosphate dehydrogenase. DHAP is a substrate of all three enzymes. In addition to their synthesis, this manuscript describes the stability of haloacetol phosphates and their reactivity toward free amino acids. The subsequent paper in this series reports the reaction of haloacetol phosphates with fructose diphosphate aldolase.

Iodoacetol phosphate has been reported to be an active-site specific reagent for triose phosphate isomerase (Hartman, 1968).

Experimental Procedure

Materials. 3-Chloropropanediol, epibromohydrin, trimethyl orthoformate, DCC, and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Aldrich Chemical Co. Other materials and vendors were glycerophosphate dehydrogenase and DHAP, Sigma Chemical Co.; glutathione and oxidized glutathione, Schwarz BioResearch; *p*-HMB, Mann Research Laboratories. Acetol phosphate was prepared by the method of Sellinger and Miller (1958).

Methods. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Melting points are uncorrected.

Unless otherwise indicated, concentrations were performed by using a rotary evaporator at 50° and water-aspirator pressure.

Thin-layer chromatography was conducted on MN-Polygram (precoated plastic sheets distributed by Brinkmann Instruments, Inc.) of the types Sil N-HR and Cel 300, both containing a fluorescent indicator. Paper chromatography was conducted by the descending method on Whatman No. 1 paper. Solvent systems used were as follows: solvent 1, ether–petroleum ether (bp 30–60°) (1:1, v/v); and solvent 2, butanol–glacial acetic acid–water (7:2:5, v/v). Phosphate esters were detected with acid molybdate spray (Hanes and Isherwood, 1949). Other methods of detection used as applicable to various types of compounds were ultraviolet light, ninhydrin, and 2,4-dinitrophenylhydrazine spray (Gray, 1952).

Phosphate was assayed by the method of Marsh (1959). Total phosphate was determined after digestion for 2 hr with 1 M sulfuric acid at 180° on a sand bath, and base-labile phosphate after 10 min in 1 N NaOH at room temperature.

Iodide was oxidized with nitrite to iodine which was measured by titration with thiosulfate (Kolthoff and Sandell, 1948). Iodide was also measured spectrophotometrically at 240 nm by using ϵ 4200.

Glutathione solutions were standardized by measuring SH concentration according to Ellman (1959). The ninhydrin color yield of glutathione, relative to leucine, as measured on the amino acid analyzer was 0.67. (Moore and Stein (1948) reported 0.76.)

Oxidized glutathione solutions were standardized by

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[1] Abbreviations used in this work are: DHAP, dihydroxyacetone phosphate; CAP, chloroacetol phosphate; BAP, bromoacetol phosphate; IAP, iodoacetol phosphate; *p*-HMB, *p*-hydroxymercuribenzoate; DCC, dicyclohexylcarbodiimide; GSH, glutathione; GSSG, oxidized glutathione.

subjecting aliquots to amino acid analysis after hydrolyzing m sealed, evacuated (50 μ) tubes with 6 N HCl for 21 hr at 110°. The ninhydrin color yield of oxidized glutathione, relative to leucine, was 0.90. (Moore and Stein (1954) reported 0.93.)

Concentrations of haloacetol phosphates were determined as base-labile phosphate. Corrections were made for any P_i and DHAP present.

DHAP was assayed enzymatically with glycerophosphate dehydrogenase (Bücher and Hohorst, 1963). The final volume of the assay medium was 3.0 ml containing 0.05 M triethanolamine hydrochloride (pH 8.0), 0.15 mM NaDH, 30 μg of glycerophosphate dehydrogenase, and 0.05–0.25 μmole of DHAP. At 340 nm, 0.1 μmole of DHAP caused a decrease in absorbancy of 0.207 optical density unit.

Stability of Haloacetol Phosphates. The release of iodide from IAP was measured by monitoring the increase in *A* at 240 nm. Release of chloride and bromide from CAP and BAP, respectively, was measured with an Orion, halide-specific electrode. The rates of decomposition of haloacetol phosphates at pH 10.2 were also measured by assaying for DHAP formed as described.

Reactivity of Haloacetol Phosphate toward Amino Acids. Mixtures of the amino acids commonly found in proteins, cysteine excluded, were prepared in 0.2 M sodium phosphate (pH 6.0), 0.2 M sodium bicarbonate (pH 8.1), and 0.2 M sodium borate (pH 10.0) and were incubated with haloacetol phosphates at room temperature for 12 hr. Each amino acid was present at a final concentration of 1 mM and the haloacetol phosphate at 20 mM. After the incubation period, the reaction mixtures and control samples were subjected to amino acid analysis with a Beckman Model 120 C analyzer by the accelerated method of Spackman *et al.* (1958). The ability of haloacetol phosphates to react with cysteine was determined by sulfhydryl determinations with *p*-HMB as described by Boyer (1954).

Reaction of Haloacetol Phosphates with Glutathione. Products of the reaction between haloacetol phosphate and glutathione were characterized by ion-exchange chromatography on the amino acid analyzer and by paper chromatography. Glutathione modified by BAP was isolated by gel filtration on Sephadex G-10 and subsequent precipitation as a barium salt. Quantitative sulfhydryl determinations with *p*-HMB were performed to measure the rate of reactions between glutathione and CAP or BAP. The rate of reaction between glutathione and IAP was determined by monitoring iodide release at 240 nm.

Synthesis of Haloacetol Phosphates

1-O-Benzoyl-3-chloro-2-propanone (*I*). To a vigorously stirred, precooled (−20°) solution of 3-chloropropanediol (110 g, 1 mole) in 600 ml of anhydrous pyridine was added dropwise 117 ml (1 mole) of benzoyl chloride. During the addition, which required 2 hr, the temperature never exceeded −10°. The reaction mixture was then warmed to 4° in an ice bath, and after 4 hr the excess pyridine was removed by concentration. The residue was dissolved in chloroform (500 ml); the resulting solution was extracted twice with 1-l. ice-cold portions of 1 N sulfuric acid, saturated aqueous sodium bicarbonate, and water in succession. The chloroform solution was dried over anhydrous sodium sulfate, filtered, and concentrated to dryness. The syrupy residue (205 g) consisted of two ultraviolet-absorbing components R_F 0.28 and 0.51 (thin-layer chromatography on Sil N-HR with solvent 1). The material with R_F 0.28 comprised an estimated 75% of the mixture and was presumed to be predominantly 1-*O*-benzoyl-3-chloropropanediol; the other component was probably 1,2-di-*O*-benzoyl-3-chloropropanediol.

The mixed benzoates of 3-chloropropanediol were oxidized by one method of Pfitzner and Moffatt (1965). A mixture containing the benzoate esters (50 g), dimethyl sulfoxide (20 ml), pyridine (3 ml), and DCC (90 g) in 400 ml of ether was cooled to 4° in an ice bath. The mixture was then removed from the ice bath, and the oxidation was initiated by the addition of 3 ml of trifluoroacetic acid to the efficiently stirred ethereal solution. The temperature rose to 35° within 10 min. Thirty minutes after the addition of trifluoroacetic acid, 20 g of oxalic acid in 30 ml of methanol was added during 15 min to decompose the excess DCC. Thirty minutes later the dicyclohexylurea was removed by suction filtration, and the filtrate was extracted three times with 200-ml portions of cold, saturated sodium bicarbonate. The ether layer was dried over sodium sulfate, filtered, and concentrated to give a crystalline mass, which was then dissolved in 300 ml of boiling cyclohexane. The small amount of insoluble material remaining was removed by gravity filtration. During cooling at room temperature for 8 hr, 29 g of crystals was deposited. To remove traces of dicyclohexylurea, the material was dissolved in 600 ml of ether, and the insoluble substituted urea was removed by suction filtration through Celite. The ether was evaporated, and the product was recrystallized from 500 ml of cyclohexane to yield 23.5 g of pure 1-*O*-benzoyl-3-chloro-2-propanone, mp 93.5–95.5°. *Anal.* Calcd for $C_{10}H_9ClO_3$ (212.64): C, 56.48; H, 4.26; Cl, 16.68. Found: C, 56.71; H, 4.46; Cl, 16.90.

1-O-Benzoyl-3-bromo-2-propanone (*II*). This compound was prepared from 3-bromo-1,2-propanediol (Winstein and Goodman, 1954) by the procedure described for preparation of the corresponding chloro derivative and melted at 84–86°. Graham (1966) prepared the same compound by bromination of 1-*O*-benzoyl-2-propanone and reported a melting point of 82–85°. *Anal.* Calcd for $C_{10}H_9BrO_3$ (257.10): C, 46.72; H, 3.53; Br, 31.08. Found: C, 46.94; H, 3.49; Br, 31.30.

1-O-Benzoyl-3-iodo-2-propanone (III). Sodium iodide (12 g, 80 mmoles) in 25 ml of acetone was added to a solution of 60 ml of acetone containing 12 g (57 mmoles) of I. Sodium chloride began to precipitate immediately, and after 30 min 3 g (93%) was removed by filtration. The acetone was evaporated at 25°, and the residue was dissolved in 100 ml of chloroform. The chloroform solution was washed three times with 100-ml portions of ice-cold water, dried over sodium sulfate, filtered, and concentrated to give a crystalline residue that was dissolved in 70 ml of benzene. Addition of 100 ml of petroleum ether and cooling at 4° resulted in deposition of 11.4 g of III. An additional 2.4 g was obtained upon concentration of the mother liquor; total yield 13.8 g (80%). A sample recrystallized from the same solvent for elemental analysis melted at 79–80°. *Anal.* Calcd for $C_{10}H_9IO_3$ (304.09): C, 39.49; H, 2.98; I, 41.47. Found: C, 39.64; H, 3.03; I, 41.68.

1-O-Benzoyl-3-iodo-2-propanone Dimethyl Ketal (*IV*). A mixture of 25 g of III in 250 ml of methanol–trimethyl orthoformate (2:3) containing 1.5 ml of concentrated sulfuric acid was left at room temperature for 24 hr, at which time thin-

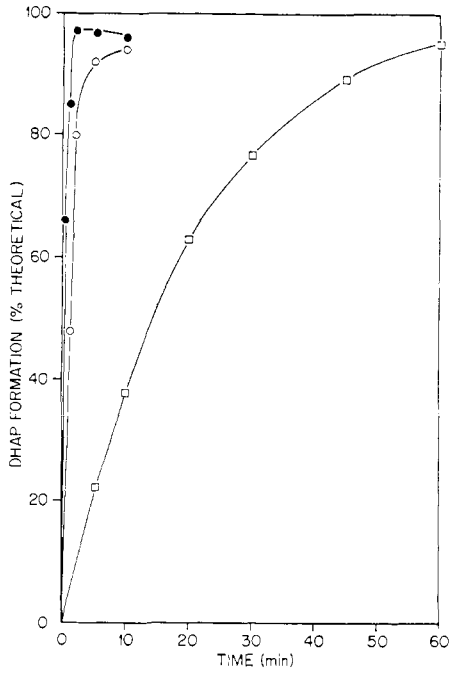


FIGURE 1: Time course of DHAP formation from haloacetol phosphates at 24°. Solutions of CAP (□), BAP (●), and IAP (○), all at 0.025 M, were incubated in 0.25 M sodium carbonate (pH 10.2). Periodically, 10-μl aliquots were assayed for DHAP as described in the Methods section. The values plotted for CAP represent the sum of DHAP and P_i formation.

layer chromatography (Sil N-HR, solvent 1) revealed the complete absence of starting material, R_F 0.29, and the appearance of a single, new component, R_F 0.53. The reaction mixture was neutralized with solid potassium carbonate, filtered, and concentrated to dryness. The remaining syrup was dissolved in 200 ml of chloroform, and the resulting solution was washed once with 250 ml of cold, saturated sodium bicarbonate solution. The chloroform layer was dried and concentrated to yield 27 g (97%) of syrupy IV.

1-Hydroxy-3-iodo-2-propanone Dimethyl Ketal (V). Compound IV (75 g, 71.4 mmoles) was dissolved in 200 ml of methanol, and to this solution was added 25 ml of 4 N sodium hydroxide. After the reaction mixture had remained at room temperature for 6 hr, the methanol was evaporated, and the remaining aqueous mixture was extracted twice with 150-ml portions of ether. The extracts were then dried and concentrated to give 15.5 g (84%) of V as a slightly viscous, light yellow liquid. This material migrated as a single component, R_F 0.20, during thin-layer chromatography (Sil N-HR, solvent 1) and was visualized by spraying with 2,4-dinitrophenylhydrazine reagent followed by heating at 100° for 3 min.

1-Hydroxy-3-iodo-2-propanone Phosphate Dimethyl Ketal Biscyclohexylammonium Salt (VI). Compound V (10 g, 40.7 mmoles), dissolved in 100 ml of tetrahydrofuran, was added during 1 hr to an efficiently stirred solution at −2° of 300 ml of tetrahydrofuran containing phosphorus oxychloride (5.5 ml, 60 mequiv) and pyridine (9.17 ml, 120 mequiv). After the addition was completed, the reaction mixture was stirred an additional 30 min at which time 200 ml of cold (4°) 0.3 M aqueous pyridine was added with vigorous agitation. The

TABLE I: Half-Lives of Haloacetol Phosphates.[a]

Reagent	Half-Life (hr) at pH		
	6.0 (0.1 M Sodium Cacodylate)	7.0 (0.1 M Na_2HPO_4)	8.0 (0.1 M $NaHCO_3$)
BAP	7.2	6.0	2.5
IAP	8.5	6.8	3.0

[a] Solutions of IAP (0.4 mM) and BAP (1.0 mM) were incubated at room temperature. Iodide and bromide release were monitored as described in Methods section.

tetrahydrofuran was evaporated at 40°, and the remaining aqueous solution was passed through a 3 × 50 cm column of Dowex 50 W-X4 (cyclohexylammonium form, 50–100 mesh). The eluate was concentrated to dryness after its pH had been adjusted to 8.5 with cyclohexylamine. The last traces of water were removed by two concentrations from 300 ml of ethanol. Isopropyl alcohol–ethyl acetate (200 ml of a 1:1 mixture) was added to the residue, and after cooling the slurry in an ice bath, the insoluble P_i and desired phosphate ester (combined total of 20.2 g) were collected by filtration and suspended in 400 ml of ethanol. After refluxing this mixture for 30 min, the insoluble P_i (8.0 g) was removed. Addition of 200 ml of ether to the filtrate followed by cooling to 4° gave 11.0 g of crystalline VI. Recrystallization by dissolving in 50 ml of water followed by addition of 150 ml of acetone and cooling at 4° yielded 9.8 g (43%) of chromatographically pure material, R_F 0.77 (paper with solvent 2). The compound contained 1.00 ± 0.03 molar equiv of total phosphate and was contaminated with 0.01 molar equiv of P_i. *Anal.* Calcd for $C_{17}H_{38}IN_2O_6P \cdot 2H_2O$ (558.41): C, 36.56; H, 7.58; I, 22.72; N, 5.02; OCH_3, 11.29; P, 5.55. Found: C, 36.57; H, 7.47; I, 22.49; N, 5.02; OCH_3, 10.83; P, 5.77.

1-Hydroxy-3-iodo-2-propanone Phosphate (IAP). A solution of 112 mg (0.2 mmole) of VI in 4.0 ml of water (0.05 M) was stirred for 5 min with 2 mequiv of Dowex 50 (H^+), filtered, and incubated at 40°. The half-time for conversion into the free ketone was 5.5 min. After 30 min, the solution was 0.05 M in alkali-labile phosphate and iodide (1 N NaOH for 10 min at room temperature). No detectable release of inorganic phosphate or iodide resulted during the incubation. Paper chromatography with solvent 2 revealed a single phosphate ester, R_F 0.50. The solution was stored frozen after raising the pH to 4.5 with solid sodium bicarbonate.

3-Bromo and 3-Chloro Derivatives of 1-Hydroxy-2-propanone Phosphate (BAP and CAP). The dimethyl ketals of these phosphate esters were synthesized from the appropriate 1-*O*-benzoyl-3-halo-2-propanone according to the procedure described for the iodo derivative and crystallized as biscyclohexylammonium salts; R_F values in solvent 2 on paper: CAP dimethyl ketal, 0.73; BAP dimethyl ketal, 0.75. *Anal.* Calcd for $C_{17}H_{38}ClN_2O_6P \cdot 2H_2O$ (468.97): C, 43.54; H, 9.03; Cl, 7.56; N, 5.97; P, 6.60. Found: C, 43.71; H, 8.94; Cl, 7.42; N, 5.77; P, 6.76. *Anal.* Calcd for $C_{17}H_{38}BrN_2O_6P \cdot 2H_6O$ (513.43): C, 39.77; H, 8.25; Br, 15.57; N, 5.46; P, 6.03. Found: C, 40.02; H, 8.55; Br, 15.48; N, 5.33; P, 6.18.

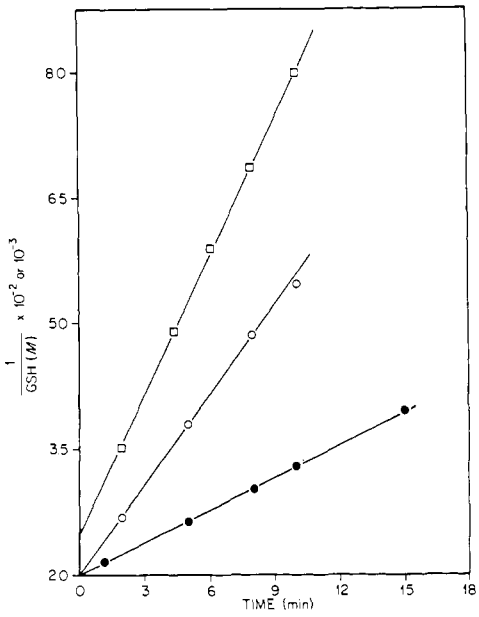


FIGURE 2: Kinetics of the reactions of haloacetol phosphates with GSH at 24°. The reaction of CAP (5 mM) with GSH (5 mM) was conducted in 0.1 M sodium acetate, pH 4.0 (●); the reaction of BAP (5 mM) and GSH (5 mM) was conducted in 0.1 M sodium phosphate, pH 7.0 (○). Both reactions were monitored by periodically transferring 50-μl aliquots to cuvets containing 2.45 ml of 0.15 mM *p*-HMB (dissolved in 0.2 M sodium acetate (pH 5.5), containing 2 M ammonium sulfate) and determining the $A_{255\ nm}$. The reaction of IAP (0.4 mM) with GSH (0.4 mM) was conducted in 0.1 M sodium acetate (pH 4.0) (□) and the rate was determined by observing iodide formation at 240 nm.

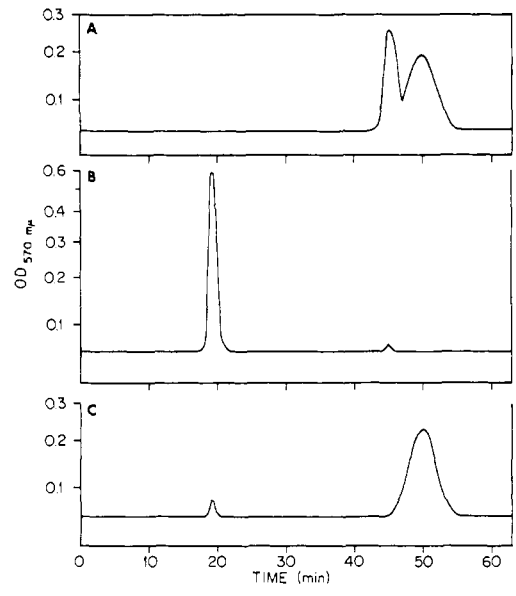


FIGURE 3: Chromatograms from the amino acid analyzer of (A) a standard mixture containing 0.1 μmole of GSH and 0.05 μmole of GSSG, (B) 0.1-μmole sample of GSH after modification with CAP or BAP, and (C) 0.1-μmole sample of GSH after treatment with IAP.

The ketals were hydrolyzed to the ketones by incubating 0.05 M solutions of the free acids at 40°; the half-times for formation of BAP and CAP were 6.5 and 14.3 hr, respectively. After 40 hr, the solution of the chloro compound was 3.4 mM in P_i, 39.4 mM in base-labile phosphate, and 50 mM in total phosphate. The solution of the bromo compound was 4.8 mM in P_i, 47 mM in base-labile phosphate, 51 mM in total phosphate, 2.0 mM in DHAP, and 4.2 mM in bromide. The R_F values determined by paper chromatography with solvent 2 were 0.40 for CAP and 0.42 for BAP. After adjusting the pH to 4.5 with solid sodium bicarbonate, the solutions of CAP and BAP were stored frozen.

Results

Loss of Halide from Haloacetol Phosphates. The time course of DHAP formation from haloacetol phosphates at pH 10.2 is illustrated in Figure 1. Half-lives for bromide and iodide release from BAP and IAP, respectively, at pH 6.0, 7.0, and 8.0 are given in Table I; CAP did not decompose at a measurable rate.

Reactivity of Haloacetol Phosphates toward Amino Acids. Incubation at pH 6.0, 8.0, and 10.0 of mixtures containing tryptophan, lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine with haloacetol phosphates as described in the Methods section did not result in a detectable loss of any amino acid. Under the same conditions, incubation of cysteine with haloacetol phosphates resulted in a rapid (<1 min for BAP and IAP) and complete loss of free SH. Tryptophan, lysine, histidine, methionine, and tyrosine were also incubated individually with haloacetol phosphates, but no reaction could be detected.

Reaction of CAP and BAP with Glutathione. The second-order rate constants at 24° for the reactions between glutathione and CAP at pH 7.0 and BAP at pH 4.0 are 0.22 and 0.60 M^{-1} sec^{-1}, respectively (Figure 2). After completion of the reactions, paper chromatography reveals a single ninhydrin-positive phosphate ester (R_F 0.27, solvent 2), which emerges from the long column of the amino acid analyzer at 19 min (Figure 3).

Isolation and Characterization of the Product from the Reaction of Glutathione with BAP. Glutathione (0.3 mequiv) was modified with BAP; a stoichiometric release of protons accompanied the reaction (Figure 4). The entire reaction mixture was placed on a 2 × 85 cm column of Sephadex G-10 equilibrated with water. Modified glutathione emerged at 90–110 ml (flow rate = 10 ml/hr). Barium chloride (300 mg) was added to the pooled fractions, and the barium salt of the glutathione derivative was precipitated by addition of absolute ethanol (10 ml). The precipitate was collected by centrifugation, washed thoroughly with 50% aqueous ethanol followed by absolute ethanol, and dried; obtained 170 mg (89% based on a molecular weight of 662 for the barium salt of the presumed *S*-alkyl derivative).

The isolated glutathione derivative, chromatographically indistinguishable from the derivative in the initial reaction mixture, contained 1.05 molar equiv of total phosphate, 9% of which was present as P_i. The phosphate moiety was base stable but hydrolyzed by 1 M sulfuric acid at 100° (60% of the organic phosphate released in 10 min). On the basis of ele-

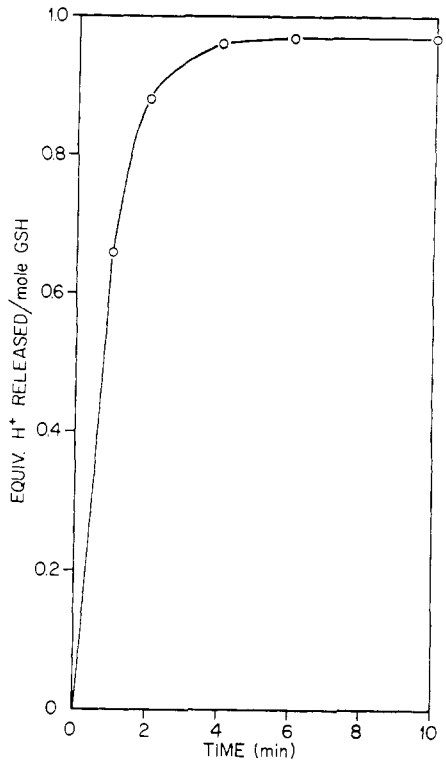


FIGURE 4: Proton release during modification of GSH by BAP. Six milliliters each of GSH (0.05 M) and BAP (0.05 M), both solutions at pH 6.0, were combined, and the quantity of 0.1 N sodium hydroxide required to maintain pH 6.0 was determined.

mental analysis, the glutathione derivative did not contain bromine.

Reaction of IAP with Glutathione. Chromatography on the amino acid analyzer of glutathione samples that were treated with IAP reveals oxidized glutathione as the major, ninhydrin-positive product (Figure 3). Two moles of SH is oxidized by one mole of IAP as determined by iodide released (Figure 5). The yields of oxidized glutathione obtained with varying molar ratios of IAP to glutathione are given in Table II. The

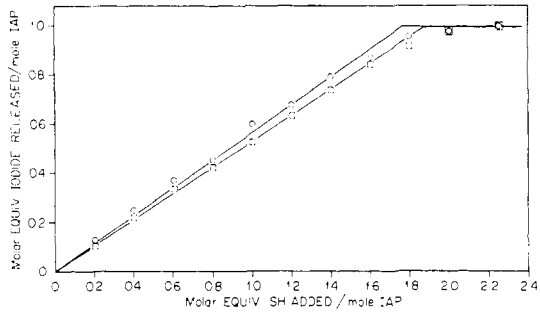


FIGURE 5: Stoichiometry of the oxidation of thiols by IAP. Aliquots (10 μl) of 0.02 M solutions of GSH (○), cysteine (□), or β-mercaptoethanol (□) (values obtained with cysteine and β-mercaptoethanol coincided) were added to cuvets containing 0.4 mM IAP in 0.1 M sodium phosphate (pH 7.0). Two minutes after each addition, the quantity of iodide formed was calculated from the $A_{240\ nm}$. Corrections were made for the A attributable to disulfide.

TABLE II: Yields of GSSG Formed with Varying GSH:IAP Molar Ratios.[a]

GSH (mM)[b]	IAP (mM)[b]	GSH:IAP	GSSG (% Yield)
10	1	10	100
4	1	4	94
2	1	2	90
1	1	1	90
1	10	0.1	90
0.5	50	0.01	88

[a] All reactions were conducted in 0.1 M sodium phosphate (pH 7.0) at room temperature. Five minutes after initiation of the oxidation, the reaction mixtures were acidified to pH 2.0 and an aliquot was analyzed with the amino acid analyzer. [b] Final concentration in reaction mixture.

second-order rate constant at pH 4.0 and 24° for the oxidation of glutathione by IAP is 9.1 M^{-1} sec^{-1} (Figure 2).

Reaction of IAP with Cysteine. IAP (7 ml of a 0.05 M solution, pH 6.0) was added to L-cysteine (2 ml of a 0.25 M solution, pH 6.0); cystine began to precipitate immediately, and after 5 min the reaction mixture was cooled in an ice bath. The cystine (51 mg, 85%) was collected by filtration; $[\alpha]_D^{25}$ −210° (*c* 1, 1 N HCl); $[\alpha]_D^{25}$ −212° (*c* 1, 1 N HCl) has been reported (Greenstein and Winitz, 1961).

An additional 30 mg of cysteine was added to the filtrate to react with the remaining IAP. After removal of the precipitated cystine, several experiments were performed to characterize the phosphate ester formed during the oxidation of cysteine. Paper chromatography demonstrated a single phosphate ester (visualized with acid molybdate and 2,4-dinitrophenylhydrazine), whose R_F (0.41 in solvent 2) was identical to that of acetol phosphate. Phosphate analyses on the filtrate containing the presumed acetol phosphate indicated the solution to be 38 mM total phosphate but only 2 mM in base-labile phosphate. The filtrate gave a positive iodoform test. An aliquot (5 μl, 0.19 μmole of phosphate ester) was added to a cuvet containing 0.15 mM NADH and 1 mg of glycerophosphate dehydrogenase in 3.0 ml of 0.05 M triethanolamine hydrochloride (pH 8.0). A slow decrease in $A_{340\ nm}$ occurred and was completed in 2 hr (total ΔA is 0.42; 105%).

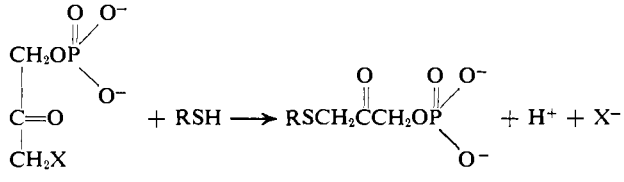
Discussion

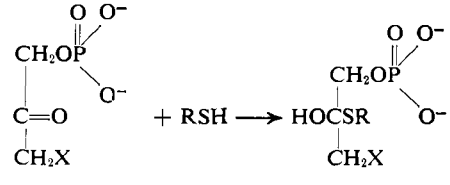
Although the haloacetol phosphates, prepared according to Scheme I, were never isolated from solution, several lines of evidence support their formation: (1) The immediate precursors of haloacetol phosphates, their dimethyl ketals, were isolated as crystalline materials with the calculated elemental composition. (2) Mild acid treatment of the ketals gave a single new phosphate ester as shown by paper chromatography. (3) During conversion of the ketals into the free ketones, equivalent quantities of base-labile halogen and phosphate appeared. (4) Mild base treatment of the presumed haloacetol phosphates gave 95% yields of DHAP.

The stability and reactivity toward model compounds of haloacetol phosphates were studied since such information can be of value in designing protein modification experiments and can indicate which amino acid side chains in proteins are potential sites of modification. In carbonate buffer (pH 10.2), BAP and IAP are converted quantitatively into DHAP within 5 min (Figure 1). Under the same conditions, CAP has a half-life of 15 min (Figure 1). The quantity of DHAP formed from CAP is less than the theoretical value owing to the lability of the phosphate group of DHAP at pH 10.2 ($t_{1/2}$ = 75 min). Thus the sum of the concentrations of P_i and DHAP must be used to calculate CAP disappearance.

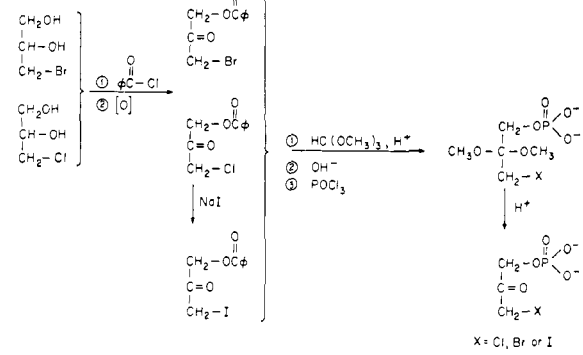
Haloacetol phosphates are much more stable at lower pH (Table I). During 3 hr at pH 6–8, no significant decomposition of CAP was detected. In contrast to the decomposition of haloacetol phosphates at pH 10.5, their decomposition at pH 6–8 does not result in the formation of an equivalent amount of DHAP. In general, the yield of DHAP is only 0–5%. A possible explanation is that the halide is displaced intramolecularly by the phosphate anion to form a six-membered cyclic phosphate. Attempts to convert this hypothetical cyclic phosphate into DHAP by acid, base, or various phosphatases have failed.

Since the SH group of cysteine was the only functional group of free amino acids which reacted with haloacetol phosphates, the reaction of these reagents with glutathione, a reasonable model for protein SH groups, was studied in detail. The product of the reaction between glutathione and CAP or BAP emerged from the long column of the amino acid analyzer at the position of cysteic acid (19 min) (Figure 3). No other ninhydrin-positive compounds were detected. Based on the initial concentration of glutathione and the assumption that all of the reacted glutathione is accounted for by the single, ninhydrin-positive component, its color yield relative to leucine is 0.95. Paper chromatography revealed a component with R_F 0.27 (solvent 2) that was ninhydrin positive and contained organic phosphate. Thus CAP and BAP react with glutathione to form an addition product. Two types of reactions can be visualized: an alkylation of the sulfhydryl group or the formation of a hemithioketal, which can occur under mild conditions (Schubert, 1936). Present evidence favors the alkylation reaction. A stoichiometric release of protons accompanies the reaction of BAP with glutathione (Figure 4), and an isolated sample of the product contains no bromine. Indirect evidence also suggesting an alkylation is the observation that glutathione and DHAP do not form an addition compound.





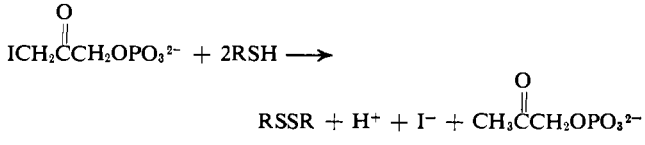
SCHEME I



The only major ninhydrin-positive component in reaction mixtures of glutathione and IAP was oxidized glutathione (Figure 3). A small peak, 5% as large as the oxidized glutathione peak, eluted at 19 min. Amino acid analysis also demonstrated cystine to be the only ninhydrin-positive component resulting from the reaction of cysteine with IAP, and cystine was isolated in 85% yield.

The phosphate ester formed from IAP during the oxidation of sulfhydryl compounds is acetol phosphate. Several observations are consistent with this conclusion. The phosphate group of the product is stable to base, as is the phosphate group of acetol phosphate (Sellinger and Miller, 1958; Rose and O'Connell, 1969). The product and an authentic sample of acetol phosphate are not separated by paper chromatography (R_F 0.41 in solvent 2). The product reacts with 2,4-dinitrophenylhydrazine and gives a positive iodoform test demonstrating a methyl ketone structure. Finally, a commercial preparation of glycerophosphate dehydrogenase catalyzes the reduction of the presumed acetol phosphate by NADH. Glycerophosphate dehydrogenase is known to contain a contaminating enzyme that catalyzes the reduction of acetol phosphate (Sellinger and Miller, 1959).

The rate of the oxidation of sulfhydryls by IAP, which is conveniently measured by monitoring the release of iodide spectrophotometrically at 240 nm, is extremely rapid. At pH 7.0, an essentially instantaneous release of iodide is observed when thiols are added to a solution of IAP. During the oxidation, about 0.5 mole of iodide/mole of sulfhydryl compound is formed (Figure 5). Thus, the over-all reaction for the oxidation of sulfhydryls to disulfides is



Since the observed molar ratio of iodide to sulfhydryl compound was somewhat larger than 0.5 (Figure 5) and suggested that other products are formed as free sulfhydryl becomes

limiting, the yields of disulfide as a function of molar ratios of glutathione to IAP were determined with the amino acid analyzer (Table II). When glutathione was in large excess ([GSH]/[IAP] = 10), the yield of disulfide, based on the initial IAP concentration, was quantitative. At [GSH]/[IAP] ratios in the range of 0.01–4, the yield of disulfide was reduced to 88–94%, but the only other ninhydrin-positive component emerged at 19 min. The significance of this minor component (Figure 3) is unknown since it was present even when quantitative yields of oxidized glutathione were observed.

I have not elucidated the mechanism of the IAP oxidation of sulfhydryls to disulfides, but presumably at least two steps are involved: an initial activation of a SH group followed by its condensation with a second SH group to form the disulfide. The activation reaction must be rate limiting since high yields of disulfide are obtained even if a 200-fold excess of IAP is used. If the iodine atom of IAP has positive character, the oxidation could proceed *via* an intermediate sulfenyl iodide as does the oxidation of sulfhydryls by elemental iodine (Kharasch, 1961; Kharasch *et al.*, 1966).

Based on the results of these studies, protein SH groups will be the most likely sites of modification by haloacetol phosphates. Protein SH groups should be covalently modified by CAP and BAP, but be oxidized by IAP. IAP may prove to be a useful reagent for selectively oxidizing protein SH groups since it does not oxidize or iodinate any other functional group found in proteins. The major oxidation products in proteins will not necessarily be disulfides. Tetranitromethane, which converts glutathione primarily into the disulfide (Sokolovsky *et al.*, 1966), oxidizes SH groups of aldolase to sulfinic acids (Riordan and Christen, 1968; Sokolovsky *et al.*, 1969).

References

Baker, B. R. (1967), Design of Active-Site-Directed Irreversible Enzyme Inhibitors, New York, N. Y., Wiley.

Boyer, P. D. (1954), *J. Amer. Chem. Soc. 76*, 4331.

Bücher, T., and Hohorst, H.-J. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic, p 246.

Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1969), *J. Biol. Chem. 244*, 4316.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Graham, W. (1966), *Chem. Abstr. 65*, 7109*g*.

Gray, R. A. (1952), *Science 115*, 129.

Greenstein, J. P., and Winitz, M. (1961), *in* Chemistry of the Amino Acids, Vol. 3, New York, N. Y., Wiley, p 1916.

Hanes, C. S., and Isherwood, F. A. (1949), *Nature 164*, 1107.

Hartman, F. C. (1968), *Biochem. Biophys. Res. Commun. 33*, 888.

Kharasch, N. (1961), *in* Organic Sulfur Compounds, Kharasch, N., Ed., New York, N. Y., Oxford, Pergamon, p 387.

Kharasch, N., Ariyan, Z. S., and Havlik, A. J. (1966), *Quart. Rept. Sulfur Chem. 1*, 115.

Khedouri, E., Anderson, P. M., and Meister, A. (1966), *Biochemistry 5*, 3552.

Kolthoff, I. M., and Sandell, E. B. (1948), *in* Textbook of Quantitative Inorganic Analysis, New York, N. Y., Macmillan, p 629.

Marsh, B. B. (1959), *Biochim. Biophys. Acta 32*, 357.

Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.

Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.

Pfitzner, K. E., and Moffatt, J. G. (1965), *J. Amer. Chem. Soc. 87*, 5661.

Riordan, J. F., and Christen, P. (1968), *Biochemistry 7*, 1525.

Rose, I. A., and O'Connell, E. L. (1969), *J. Biol. Chem. 244*, 126.

Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2*, 252.

Schubert, M. P. (1936), *J. Biol. Chem. 114*, 341.

Sellinger, O. Z., and Miller, O. N. (1958), *Biochim. Biophys. Acta 29*, 74.

Sellinger, O. Z., and Miller, O. N. (1959), *J. Biol. Chem. 234*, 1641.

Shaw, E., Mares-Guia, M., and Cohen, W. (1965), *Biochemistry 4*, 2219.

Singer, S. J. (1967), *Advan. Protein Chem. 22*, 1.

Sokolovsky, M., Harell, D., and Riordan, J. F. (1969), *Biochemistry 8*, 4740.

Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Winstein, S., and Goodman, L. (1954), *J. Amer. Chem. Soc. 76*, 4368.

Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1*, 1031.