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Research Article

THE INTRAHEPATIC CONJUGATION OF SULFOBROMOPHTHALEIN AND GLUTATHIONE IN THE DOG *

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In 1950 Brauer, Krebs and Pessotti (1) reported that S^{35}-labeled sodium phenoltetrabromophthalein disulfonate (more commonly known as sulfobromophthalein, Bromsulfalein or BSP) may be recovered from the bile of the dog, during constant intravenous infusion, in four fractions which are separable by column chromatography. Similar fractions, accounting for the bulk of BSP excreted and having absorption spectra like those of standard BSP, have also been detected in the bile of sheep, rat, cat and chicken and in bile produced by the isolated perfused liver of the rat (2). More recently, Meltzer, Wheeler and Cranston (3) have found that BSP in the blood and bile of the dog and man may also be fractionated by paper chromatography and that several of the fractions yield a positive ninhydrin reaction suggesting the presence of amino acid. Glutamic acid and glycine (but not cysteine) were identified by two-dimensional chromatography following 6 N hydrochloric acid hydrolysis of an eluate of the fractions. Hence they concluded that BSP is conjugated with amino acid, presumably in the liver, since the conjugates were found in the bile prior to their appearance in the blood. Subsequent work by Javitt, Wheeler, Baker and Ramos (4) has shown that cysteine is also present in the conjugates. Similar findings have been made independently by other workers (5–7) during the past year. Glycine and glutamic acid have been demonstrated in acid hydrolysates by Combes (5) and cysteine has been found in hydrobromic acid hydrolysates of BSP conjugates by Grodsky, Carbone and Fanska (6, 7). As both Combes and Grodsky have also noted, the appearance of glycine, glutamic acid, and cysteine together would strongly suggest that glutathione might be conjugated with BSP. This inference has been substantiated in the course of the studies to be presented below.

METHODS

The biliary excretion of BSP was measured in 5 trained unanesthetized fasting dogs before and after the intravenous administration either of single doses ranging from 200 to 300 mg or of BSP infusions over a prolonged period, in most instances with the establishment of high plasma BSP concentrations. Bile was collected continuously by means of an indwelling catheter placed in the common bile duct under direct vision through a Thomas duodenal cannula (8, 9). Cholecystectomy and splenectomy were performed when the duodenal fistula was prepared and the cannula inserted, several months prior to study. All bile samples were refrigerated or frozen as soon as possible after collection.

Aliquots of whole bile were applied directly to Whatman no. 1, 3 or 3 MM filter paper for one-dimensional chromatography or electrophoresis. Preliminary removal of proteins and bile salts was necessary in those specimens to be hydrolyzed for amino acid analysis. For this purpose 2 ml aliquots of bile were dialyzed in Visking cellulose tubing for 24 hours against equal volumes of distilled water. The dialysates were lyophilized to dryness, extracted three times with absolute ethanol (10) and the residue reconstituted in distilled water.

Covered glass chambers were used for one-dimensional ascending and descending chromatography with butanol: acetic acid: water (4:1:2) as the solvent mixture. For paper electrophoresis (11), a Spinco series B Durrum type apparatus was used with a constant voltage power supply. Electrophoresis was carried out at 400 v for 4 hours or 120 v for 18 hours using 5 N acetic acid (pH 1.9) as the conducting medium. Two-dimensional ascending chromatography according to Dent (12) was carried out with phenol: water (4:1) followed by 2,4-collidine: 2,4,6-lutidine: water (1:1:1) as the solvent systems. Following electrophoresis or chromatography the papers were air-dried at room temperature.

For purposes of comparison, the chromatographic and electrophoretic migration of tetrabromphenolphthalein (Eastman Organic Chemical), tetrabromphenolphthalein disulfonate (BSP, Hynson, Westcott and Dunning), so-

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dium taurocholate, and various pure amino acids (Nutritional Biochemicals Corp.), glutathione (Schwarz Laboratories), taurine (Eastman Organic Chemicals), cysteic acid, and purified BSP conjugates—dissolved in either distilled water or canine bile—were determined separately or in various combinations. The proteins present in bile were dyed on the paper with bromphenol blue (11). Amino acids, taurine, cysteic acid, and glutathione were detected by spraying with 0.1 per cent ninhydrin in water-saturated butanol or by dipping the paper into 0.1 per cent ninhydrin in acetone and heating at 100° C for 5 minutes. Bile salts were detected by Kritchevsky and Kirk's reagent (13) and tetrabromphenolphthalein, BSP, and BSP conjugates, by spraying with 15 per cent sodium carbonate or exposure to ammonia vapor. The BSP conjugates, as reported previously (3), were also found to develop color with ninhydrin. It was noted that BSP conjugates could be detected more readily at lower concentrations by the purple color appearing on alkalinization than by ninhydrin treatment. Hence, small but detectable amounts of the conjugates might be erroneously considered ninhydrin-negative.

Hydrolysis was carried out on the lyophilized dialyzed bile samples or on solutions of the various BSP conjugates prepared therefrom in the following manner. Preliminary electrophoresis was used to separate BSP and its conjugates as anions from the amino acids, pigments and other cations. The zone of the electrophoretic sheet bearing BSP conjugates (ninhydrin-positive) was cut out and eluted with water (adjusted to pH 6.0 to 6.5). The eluate was concentrated by lyophilization and applied to ascending or descending chromatograms from which the individual conjugates were cut out and eluted. Acid hydrolysis was performed in 6 N HCl at 100° C for 14 to 24 hours in sealed glass ampules. The hydrolysates were filtered, evaporated to dryness with gentle heating and air stream, and the last trace of HCl was removed *in vacuo* over KOH. Aliquots of the dried residue were dissolved in distilled water for two-dimensional chromatography. Alkaline hydrolysis was carried out in covered test tubes in 0.3 N NaOH at 60° C for 2 hours in a water bath. These hydrolysates were "desalted" electrolytically (Research Specialties Co., model 1930 A), and concentrated by evaporation prior to chromatography.

Reactive sulfhydryl groups before and after alkaline hydrolysis were detected by the sodium azide-iodine reaction described by Feigl (14). Neutralization of the alkalinized samples was necessary to prevent interference owing to formation of sodium iodide. Inorganic bromide was isolated from the synthetic reaction mixtures by paper chromatographic separation, described by Pollard and McOmie (15), using acetone: water (4:1) as a solvent. Bromide was precipitated on the paper by immersion in an ammoniacal (0.7 M NH_4OH) ethanolic (95 per cent) solution of silver nitrate (0.1 M) and "developed" by exposure to blue fluorescent light under standard conditions. The concentration could be determined quantitatively, by comparison with appropriate standard solutions of NaBr on the same chromatograms by using a Spinco analytrol. Over the range employed (0.015 to 0.03 M, 10 μl aliquots), concentration and densitometric readings showed a satisfactory linear correlation.

Glutathione in the conjugates isolated by electrophoresis and chromatography was determined by the method of Sachs and Brand (16) as glutamic acid. Since this method involves the formation of hydroxamic acid from a lactone ring, BSP itself might be expected to interfere. However, under the actual conditions employed (pH close to 7.0) it was found that BSP developed only a minimal amount of color compared with an equimolar concentration of glutathione. BSP could be recovered completely from the chromatograms by elution with a phosphate buffer solution (0.1 M, pH 10.4) and colorimetric analysis. Since glutathione did not interfere and since no change in optical density occurred in mixtures of glutathione and BSP during or after conjugation, conjugated BSP could be evaluated by reference to a standard BSP calibration curve. Glutathione labeled with S^{35} (specific activity 16.6 μc per mg) was obtained from Schwarz Laboratories. Radioautograms were obtained by placing Eastman Kodak No-screen X-ray film (in film holders) against the dried chromatogram or electrophoretogram and leaving it for 6 weeks prior to development.

RESULTS

Excretion of BSP and its conjugates in the bile of the dog. Regardless of the method of administration, biliary BSP always proved to be separable into several fractions by chromatographic analysis (Figure 1). All reacted with ninhydrin, with the exception of the fastest moving component (fraction I) which assumed the same chromatographic position as the commercially available or "standard" BSP. The ninhydrin-positive fractions varied considerably in number and concentration. As a rule, three major bands were distinguishable but, with improved chromatography, other components were detected. Fractions II and IV appeared to be homogeneous, whereas fraction III could be resolved into two additional components (a and b, Figure 1). On electrophoresis, also, biliary BSP could be separated into a number of fractions having differing mobilities (Figure 2). All moved toward the anode and the most rapidly moving component appeared again to be identical with standard BSP. When individual BSP conjugate fractions were eluted from the chromatograms and subjected to electrophoresis it was found that fractions II, IIIa and IV all migrated to the position represented by the darkest band in Figure 2. Fraction IIIb migrated in part to this position, but a major portion also appeared in the

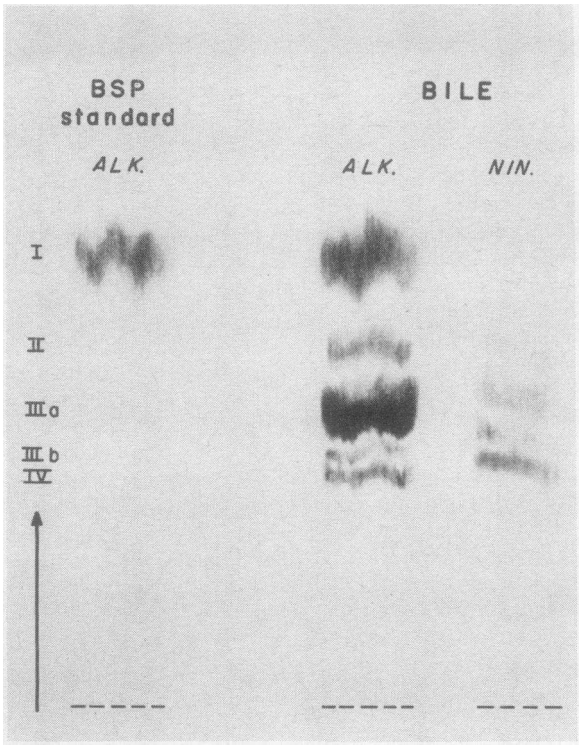


FIG. 1. CHROMATOGRAM OF SULFOBROMOPHTHALEIN (BSP) AND BSP CONJUGATES IN BILE OF THE DOG. Aliquots of solution of BSP (5 μg), BSP standard, and bile were applied at the points indicated by the broken lines. After alkalinization (Alk.) of the strips, standard BSP appeared as a single component whereas BSP appears in at least four additional fractions in bile—here labeled I, II, IIIa, IIIb, and IV for ease of reference. Fraction I corresponds to standard BSP, and does not give a positive ninhydrin reaction as do the remaining fractions (NIN). (These figures, and those that follow, have been prepared from tracings shaded in black to show relative intensities of colored bands more precisely than is possible with photographic reproduction.)

region marked by the upper ninhydrin band in Figure 2. The absence of significant hydrolysis under the conditions employed during electrophoresis was indicated by the fact that free amino acids could not be detected when purified conjugates were subjected to this procedure.

Analysis of the BSP conjugates. Acid hydrolysis of fraction IV yielded approximately equal amounts of glycine and glutamic acid. Although free BSP appeared in the reaction mixture, a large moiety of the BSP continued to migrate on chromatography as a ninhydrin-positive fraction. With alkaline hydrolysis also, large amounts of glycine and glutamic acid appeared, usually, however, in association with a small amount of a ninhydrin-positive material which behaved chromatographically like cysteine. Conversion of the relatively unstable cysteine to cysteic acid by the addition of hydrogen peroxide made it possible to demonstrate that cysteine was regularly present in alkaline hydrolysates in significant amount (Figure 3A). The azide-iodine test for reactive sulfhydryl groups, which was negative when performed on unhydrolyzed conjugates, became strongly positive after alkaline hydrolysis. These observations suggested that fraction IV is a complex of BSP and glutathione (glutamyl-cysteinyl-glycine) and that the linkage involves the sulfhydryl group of cysteine. The BSP-reacting ninhydrin-positive moiety observed after acid hy-

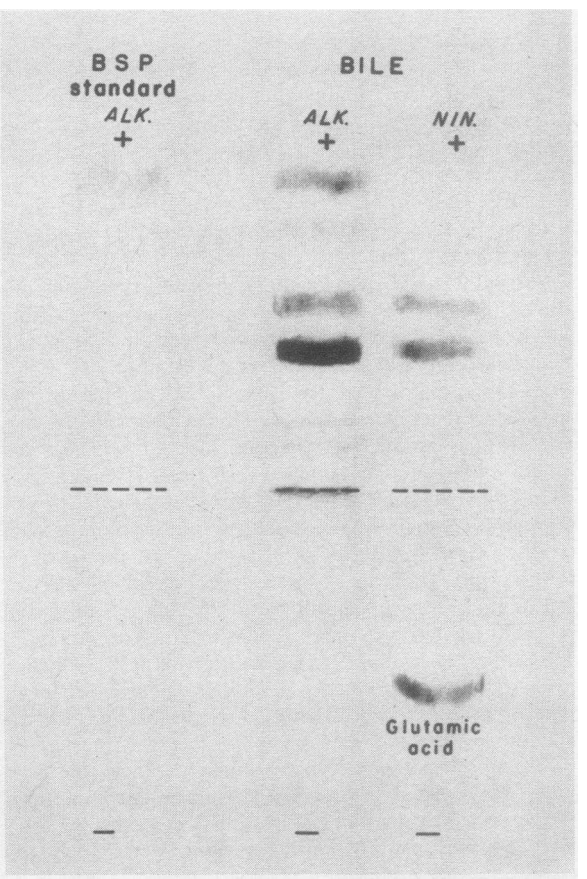


FIG. 2. ELECTROPHORETIC PATTERNS OF BSP AND BSP CONJUGATES IN BILE OF THE DOG. Standard BSP and its conjugates in the bile migrate toward the anode (+), whereas free amino acid (glutamic acid) that appears in the bile during the excretion of BSP moves toward the cathode (−) from the point of application (broken line).

drolysis of fraction IV might be a complex of BSP and cysteine.

Glycine and cysteic acid could be found following similar treatment of the chromatographically faster moving component of fraction III (Figure 3B). Glutamic acid appeared in trace amounts

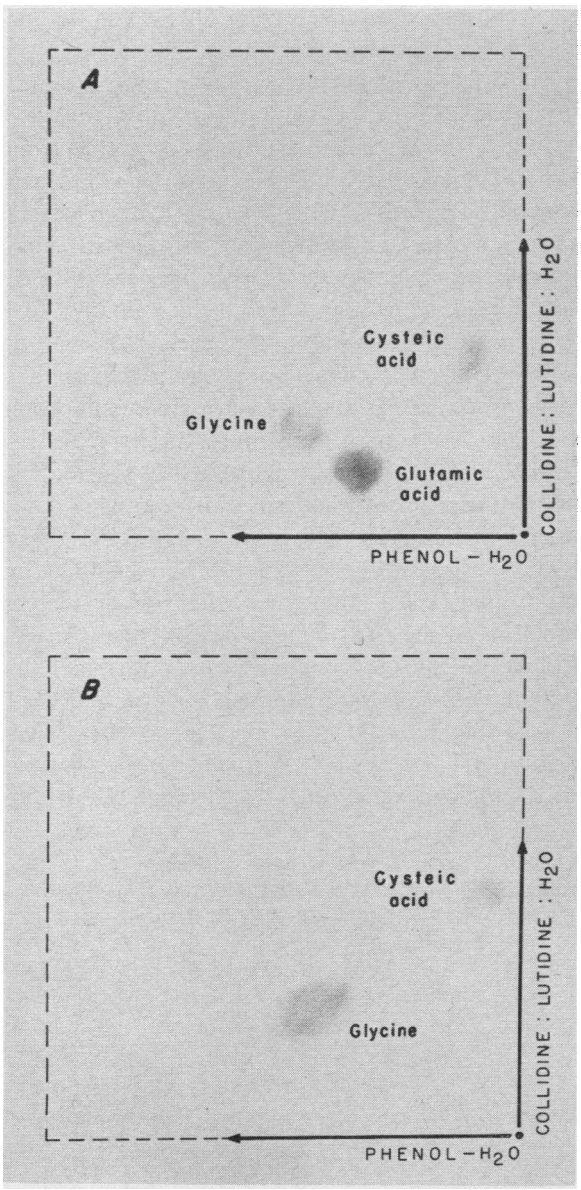


FIG. 3. CHROMATOGRAMS (TWO-DIMENSIONAL) OF ALKALINE HYDROLYSATES OF ISOLATED BSP CONJUGATES. A. Hydrolysate of fraction IV showing presence of glycine, glutamic acid, and cysteic acid following treatment with peroxide. B. Hydrolysate of fraction IIIa in which glycine and cysteic acid, but not glutamic acid, are detectable.

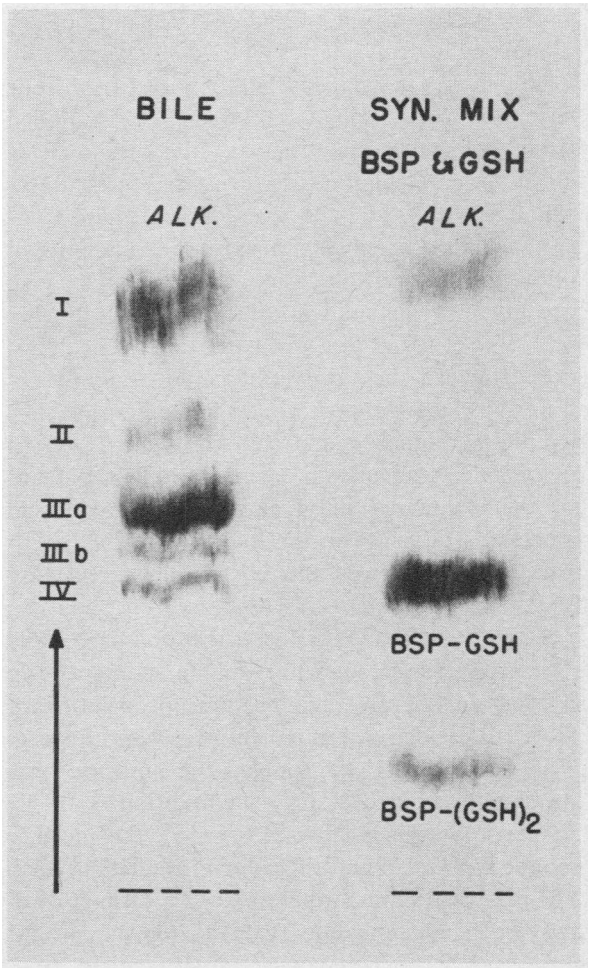


FIG. 4. CHROMATOGRAMS OF BSP AND BSP CONJUGATES IN BILE AND IN A MIXTURE OF BSP AND GLUTATHIONE. BSP and glutathione combined spontaneously in aqueous solution at 37° C to yield two detectable ninhydrin-positive conjugates. One having the chromatographic and electrophoretic properties of fraction IV appeared to be an equimolecular combination of BSP and reduced glutathione (GSH). The other, occasionally detectable in minute quantities in the bile, contained two molecules of glutathione for each molecule of BSP [BSP-$(GSH)_2$].

in early preparations but disappeared altogether as the separative procedure was improved. The more slowly moving moiety of fraction III which could be separated into two components by electrophoresis was not obtained in sufficient amount for complete analysis.

Fraction II behaved as a single substance both on chromatography and electrophoresis. Like the chromatographically faster moving portion of fraction III, it yielded glycine and cysteic acid with

appropriate treatment. Glutamic acid did not appear in the hydrolysates.

Protein-free (dialyzed) bile obtained from four normal fasting dogs not receiving BSP contained barely detectable quantities of free amino acids which were not significantly increased by hydrolysis. Eluates of the paper employed in these studies yielded on hydrolysis insignificant amounts of ninhydrin-positive material. An abundance of free glutamic acid did appear in the bile during excretion of BSP but, since the amino acid migrated toward the cathode, it could be easily removed by electrophoresis. Thus, amino acids, peptides, or other amino acid conjugates present in the bile, reagents or paper could not account for the abundance of glycine and glutamic acid which was observed in the hydrolysates of conjugates separated from the bile of dogs receiving BSP.

Synthesis of the BSP conjugates. BSP and glutathione were found to combine spontaneously *in vitro* in slightly alkaline aqueous solution at 37° C to yield two ninhydrin-positive conjugates (Figure 4). At the outset the mixture was brought to a pH of 7.3 by the addition of 0.1 N NaOH. As the reaction proceeded, the mixture became more acid and the color of the BSP faded. The pH was therefore maintained by dropwise admixture of the alkaline reagent over a 24-hour period. With exhaustion of glutathione by combination with BSP or by oxidation, the reaction came to a halt. The reaction was resumed if more glutathione was added. On both chromatography and electrophoresis two easily separable ninhydrin-positive BSP-reacting bands were demonstrable in addition to ninhydrin-negative standard BSP. Neither glycine nor glutamic acid was detectable. The major component appeared to be identical with fraction IV, chromatographically, electrophoretically and analytically. The second fraction seemed also to have its counterpart in a conjugate occasionally appearing in trace amounts in the bile of both the dog and rat.

Analysis of the eluted synthetic conjugates from glutathione and BSP indicates that the component apparently identical with fraction IV consists of an equimolecular combination of the two substances, whereas the second component (which is fluorescent in the ultraviolet) contains two molecules of glutathione for each molecule of BSP. During the reaction, hydrobromic acid is formed, accounting for the progressive acidification of the reaction mixture. Since the bromide released was found to be proportional to the amount of conjugate formed and since the conjugate contained no reactive sulfhydryl groups, BSP and reduced glutathione (GSH) probably combine by the formation of a thio-ether with replacement of bromine on the phthalic acid ring (Table I).

Cysteinylglycine prepared from pure GSH by partial hydrolysis with dilute phosphoric acid (17) also formed a conjugate with BSP *in vitro* when held at pH 7.4 and 37° C for 24 hours. Owing to the difficulty imposed by the high phosphate

TABLE I

Release of bromide during conjugation of sulfobromophthalein (BSP) and glutathione in vitro *

Exper. no.	Composition of synthetic mixture		Distribution of BSP in mixture after reaction			Amount NaOH added during reaction to keep pH constant	Computed hydrogen ion released	Observed bromide ion released
	BSP	GSH	Free	BSP-GSH	BSP-$(GSH)_2$			
	mmole			%			*mmole*	
I	0.30	0.30	52.1	43.4	4.5	0.13	0.16	0.15
II	0.42	0.42	44.4	51.4	4.2	0.21	0.25	0.24
III	0.14	0.25	22.7	68.0	9.3	0.10	0.12	0.12
IV	0.10	0.29	10.1	72.5	17.4	0.10	0.11	0.12

* In each experiment (except IV) aqueous solutions of BSP and reduced glutathione (GSH) containing the total amounts indicated under "Composition," were mixed and allowed to react at 37° C. The pH was raised to 7.3 and closely maintained by continuous titration with NaOH (0.1 N) in the total amount indicated in column 7. At equilibrium, 22.7 to 52.1% of the BSP remained unconjugated in Experiments I to III, 43.4 to 68.0% was conjugated as BSP-GSH and 4.2 to 9.3% as BSP-$(GSH)_2$. The amount of hydrogen ion which should have been released, computed as the sum of BSP-GSH and 2 × BSP-$(GSH)_2$, did not differ significantly from the amount of alkali actually added or the quantity of bromide ion released. In Experiment IV, 0.2 mmole of GSH was added to an aliquot of equilibrium mixture from Experiment II containing 0.1 mmole free BSP and 0.12 mmole conjugated BSP having the distribution shown for II. Under these circumstances the reaction came closer to completion and yielded a much larger quantity of BSP-$(GSH)_2$. Again the calculated value did not differ materially from observation.

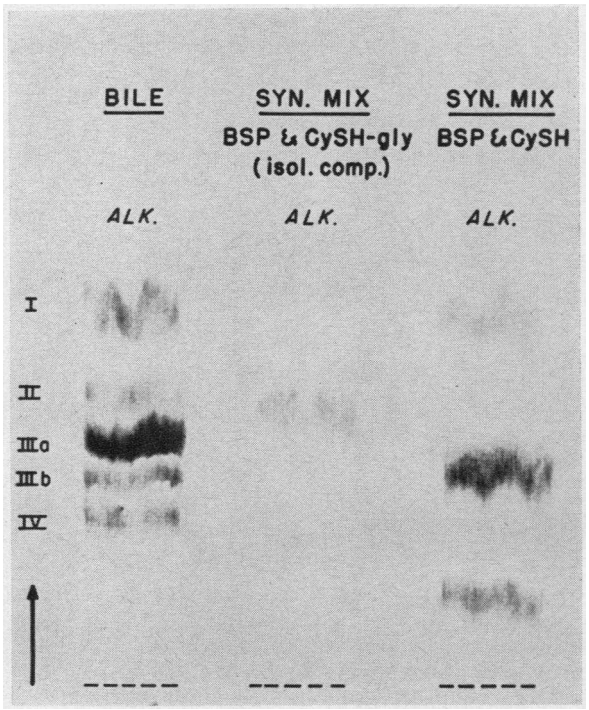


FIG. 5. CHROMATOGRAMS OF CONJUGATES PRODUCED IN AQUEOUS MIXTURES OF BSP AND CYSTEINYLGLYCINE OR CYSTEINE. Cysteinylglycine prepared from glutathione reacted with BSP to yield at least one conjugate, which behaved like fraction II when isolated from the synthetic mixture (Syn. Mix, center). At least two conjugates appeared in a mixture of BSP and cysteine, one of which resembled fraction IIIb (Syn. Mix, right).

concentration of this mixture, a preliminary chromatographic separation was necessary from which only the fastest moving portion was isolated. This material behaved electrophoretically, chromatographically (Figure 5) and analytically (two-dimensional chromatography after hydrolysis) like fraction II. Moreover, mild acid hydrolysis of fraction IV (or glutathione-BSP) resulted in the production of glutamic acid and fraction II. Thus, fraction II appears to be cysteinylglycine-BSP synthetically as well as analytically. Analysis of the faster component of fraction III indicates that it, too, is a cysteinylglycine-BSP. Cysteine and BSP have been found to combine *in vitro* to yield two conjugates of which the major seems to be identical chromatographically with the slower moiety of fraction III (Figure 5). On electrophoresis this conjugate moved to the position marked by the upper ninhydrin band in Figure 2.

Incorporation of glutathione sulfur in conjugates in vivo. Additional evidence that cysteine, derived from glutathione, appeared in the conjugates was adduced from the behavior of radioactive sulfur incorporated in glutathione during the excretion of BSP; 5 to 6 mg of glutathione labeled with S^{35} (50 to 100 μc) was injected intravenously 2 hours prior to administration of 300 mg of BSP. Bile samples were collected in the usual way and the radioactivity, in counts per minute per milliliter and BSP concentrations were determined in successive samples collected at 10- to 22-minute intervals. The biliary radioactivity and BSP concentrations rose, reached a peak, plateaued, and then fell simultaneously (Figure 6).

A one-dimensional ascending chromatogram was made from each sample and used for the preparation of a radioautograph by contact with X-ray film over a period of 6 weeks. The carbonate-sprayed chromatogram of two successive periods selected at the concentration peak, 40 minutes after the administration of BSP, and the radioautograph of the same patterns are illustrated in Figure 7. In every instance radioactive material was observed in the regions corresponding to BSP conjugates but not in the position occupied by unconjugated BSP. Since free glutathione was never detected in bile during any of the present studies, it is apparent that the S^{35} in these patterns must have been bound in the BSP conjugates.

It may be concluded, therefore, that sulfur derived from glutathione is a constant constituent of the BSP conjugates. It seems not unlikely that all of the conjugates have their origin in the combination of glutathione and BSP, fractionation resulting from the formation of isomers and from the cleavage of glutamic acid and glycine. The relatively small quantity of radioactive material detectable in the bile prior to administration of BSP was probably attributable to the excretion of breakdown products, since GSH did not appear in the bile in the absence of BSP.

DISCUSSION

The results of this study indicate that BSP and glutathione combine both *in vitro* and *in vivo* in the liver of the dog, presumably with the formation of a thio-ether linkage and release of bromine. In support of this view is the observation that a

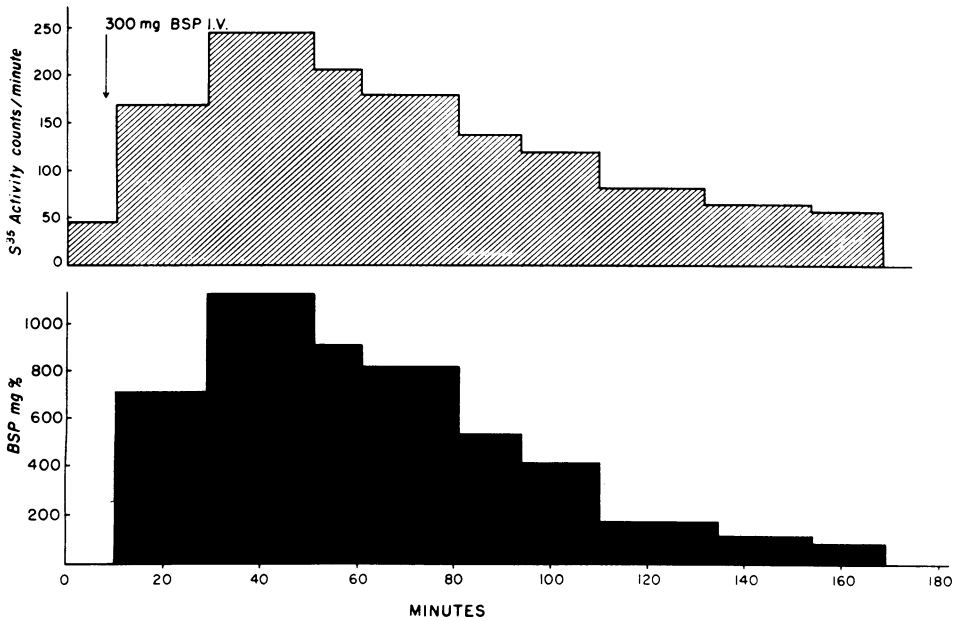
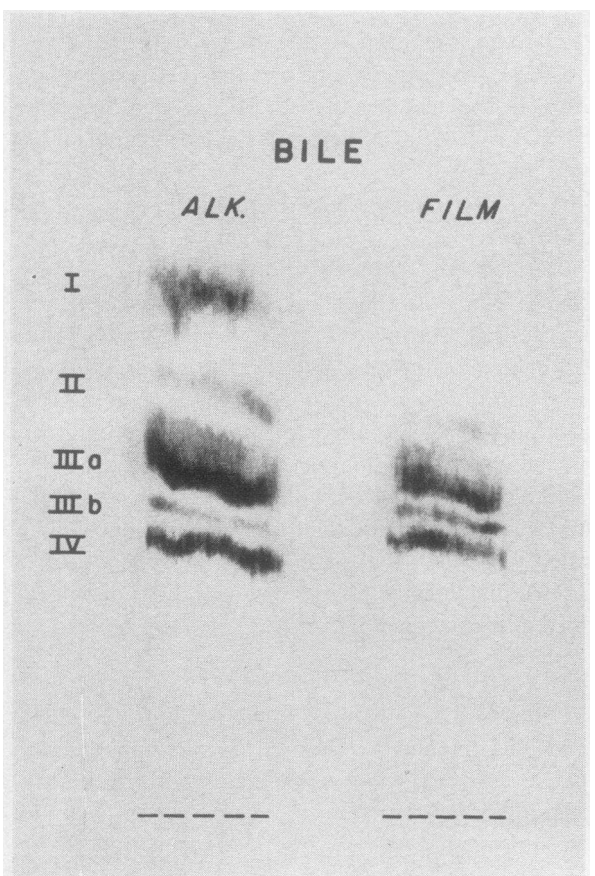


FIG. 6. BILIARY EXCRETION OF BSP AND RADIOACTIVE SULFUR IN THE DOG WHEN BSP WAS GIVEN 2 HOURS AFTER INTRAVENOUS ADMINISTRATION OF S^{35}-LABELED GLUTATHIONE. The biliary output of radioactive material increased markedly above the relatively low control value as BSP appeared in the bile. Biliary radioactivity and BSP concentration rose and fell together.



major BSP conjugate in dog bile (fraction IV in Figure 1), a ninhydrin-positive compound which yields glycine, cysteine, and glutamic acid on hydrolysis, can be synthesized by spontaneous combination of BSP with pure glutathione under conditions that preclude hydrolysis of GSH. Since an equimolar quantity of hydrogen bromide evolves during synthesis and since the conjugate possesses no free sulfhydryl groups, it may be inferred that combination involves linkage between sulfur and a carbon of the phthalic acid ring. A similar reaction has been described (18) as a means of hepatic detoxification of halogenated nitrobenzene and related compounds during the formation of mercapturic acid.

Cleavage products and isomers of a conjugate of BSP and GSH could account for all of the ninhydrin-positive, BSP-reacting substances appearing in the bile. The appearance of radioactive sulfur in all the biliary conjugates after administration of S^{35}-labeled glutathione clearly supports this conclusion. The presence of cysteinylglycine-

FIG. 7. RADIOACTIVE SULFUR IN BILIARY BSP CONJUGATES EXCRETED AFTER ADMINISTRATION OF S^{35}-LABELED GLUTATHIONE. Autoradiogram (film) of the alkalinized chromatogram of bile (Alk.); radioactivity is evident in every fraction except I (free BSP).

BSP conjugates, invariably in association with free glutamic acid, suggests that cleavage of glutamic acid may occur to some extent during excretion. The other possible products of cleavage, cysteinyl-BSP, glutamyl-cysteinyl-BSP and tribromo-BSP have not been identified. Isomerism could account for the chromatographic separation of two forms of cysteinylglycine BSP. Indeed, the appearance of a diglutathione conjugate of BSP in artificial synthetic mixtures attests to at least two sites on the BSP molecule at which combination may occur.

Regardless of the role of glutathione in BSP transport, whether essential or merely incidental, the loss of glutathione during BSP excretion could lead in principle to depletion of hepatic GSH. Such a depletion has actually been observed following the administration of a variety of substances that are excreted as mercapturic acids, but the dosages required have been much larger than those in which BSP has been administered in these or in the usual clinical procedures. Possibly a critical loss could be induced more readily in the liver, damaged by disease or hepatotoxic agents. These studies suggest that BSP may be used as a tool for the investigation of hepatic glutathione metabolism and its relation to hepatic disease.

SUMMARY

Sulfobromophthalein (BSP) and glutathione appear to combine intrahepatically in the dog to yield a variety of ninhydrin-positive conjugates. Since bromide and hydrogen ions are released stoichiometrically during synthesis of conjugates *in vitro,* it may be inferred that conjugation involves the formation of a thio-ether with replacement of bromine. Radioactive sulfur is found in all of the conjugates detectable in the bile when S^{35}-labeled glutathione is given with BSP, suggesting that all the ninhydrin-positive fractions may be derived from the conjugation of BSP and glutathione. This phenomenon may provide an approach to the quantitative investigation of hepatic glutathione metabolism in the experimental animal.

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