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*J Clin Invest.* 1959;**38**(8):1426-1433. <https://doi.org/10.1172/JCI103919>.

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# THE BILIARY EXCRETION OF SULFOBROMOPHTHALEIN SODIUM (BSP) IN THE RAT AS A CONJUGATE OF GLYCINE AND GLUTAMIC ACID\*†

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(Submitted for publication March 16, 1959; accepted April 30, 1959)

Sulfobromophthalein sodium (BSP) is a phthal-  
ein dye that is removed from blood predominantly  
by the liver and excreted into the bile (2). Re-  
moval is impaired in the presence of hepatocellular  
damage, and BSP retention in blood has proved  
to be a sensitive index of hepatic dysfunction (3,  
4). Although BSP has been used in the clinical  
evaluation of hepatic disease for over 30 years,  
the precise mechanisms by which it is handled by  
the liver remain poorly understood.

Based on a considerable body of evidence (5-  
14), it is currently believed that hepatic removal  
of BSP depends upon the simultaneous operation  
of at least two processes: 1) uptake of BSP by  
liver cells in an amount proportional to a given  
blood level, and 2) transfer from blood to bile  
by a rate-limited transfer mechanism. Recently,  
a chromatographic and chemical analysis of BSP  
as it appeared in bile was undertaken in our labo-  
ratory in order to examine the biochemical mecha-  
nisms involved in its excretion. In a preliminary  
communication (1), it was reported that BSP is  
excreted as four distinct compounds in the bile  
of the rat. The major excretory product was  
demonstrated to be a conjugate of BSP with the  
amino acids, glycine and glutamic acid. A more  
complete presentation of these and additional ob-  
servations forms the basis of the present report.

## MATERIALS AND METHODS

The standard BSP used in these studies was the 5 per cent solution sold commercially by Hynan, Westcott and Dunning, Inc., of Baltimore, Md. Chromatographically

\* A preliminary report of these studies has been previously published (1).

† This investigation was supported by research grants from the Dallas Heart Association and from the National Heart Institute of the National Institutes of Health, United States Public Health Service [H-3439].

‡ Established Investigator of the American Heart Association.

(*vide infra*), it traveled as a single band with an average  $R_f$  of 0.75.  $S^{35}$ -labeled BSP was purchased from the Volk Radiochemical Company, Chicago, Ill. The absorption spectra and extinction coefficients of the  $BS^{35}P$  and standard BSP were identical. When  $BS^{35}P$  was chromatographed, 98.4 per cent migrated with the same  $R_f$  as standard BSP (Figure 1). Two compounds accounted for the remaining 1.6 per cent, 1.3 per cent having an  $R_f$  of 0.50 and 0.3 per cent an  $R_f$  of 0.92.

The common bile ducts of rats (Long Evans, Wistar) were cannulated with fine polyethylene tubing under ether anesthesia. Bile was allowed to drain into small bottles while the rats were gently restrained in special cages. After collection of a control sample of bile, varying doses of BSP were injected and additional bile samples were obtained. The bile samples were treated as follows:

1) *Quantitation of BSP in bile.* Colorimetric determinations were made on a Beckman DU spectrophotometer, set at 575  $m\mu$ , on appropriately diluted bile specimens to which alkali had been added. Complete recovery of BSP was obtained over the range of concentrations, 30 to 350 mg. per cent, encountered in bile in these studies.

2) *Chromatography of BSP compounds.* Bile samples were applied 25  $\mu$ l. at a time as a band 5 inches wide on Whatman No. 1 filter paper strips (6 × 21 inches). Each 25  $\mu$ l. volume was dried with cold air from a hair dryer before the next aliquot was added to the paper. A total of 100 to 200  $\mu$ l. of bile containing 0.045 to 0.35 mg. of BSP was applied at the origin. The chromatograms were then developed for 16 to 20 hours at 22° C. in a descending system consisting of glacial acetic acid : water : *n*-propyl alcohol (1 : 5 : 10 v/v). After the papers were dried, BSP bands were identified by: 1) the development of a purplish color when exposed to ammonia vapors, and 2) their absorption in the ultraviolet range ( $\lambda$  max = 232  $m\mu$ ).

3) *Quantitation of BSP on chromatograms.* In some studies, each BSP band was cut out from the chromatograph; the dye was then eluted from the paper into distilled water for 30 minutes and the BSP content of the eluate measured colorimetrically on a Beckman DU spectrophotometer after the addition of alkali. The sum of the optical densities of all the bands accounted for approximately 90 per cent of the BSP originally applied on the chromatogram. For this reason, it was assumed in the following study that the 10 per cent loss of BSP was uniformly distributed among the BSP derivatives. Thus, the percentage of BSP in each band was calculated by

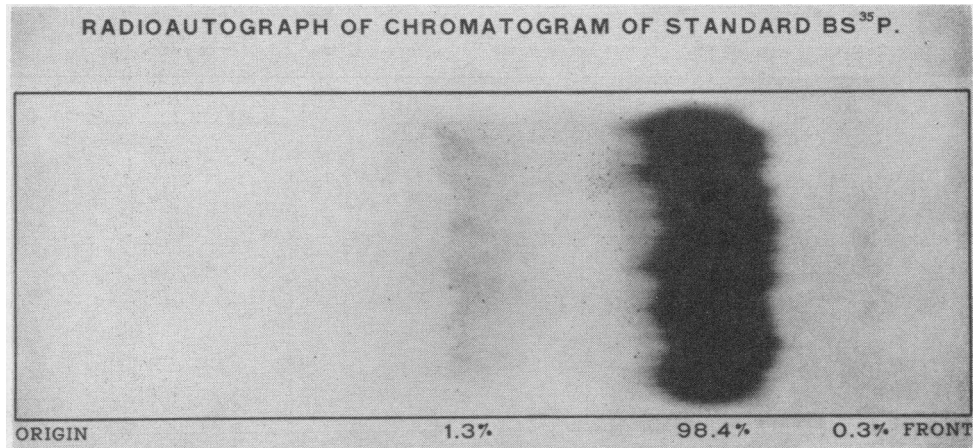


FIG. 1. RADIOAUTOGRAPH OF CHROMATOGRAM OF STANDARD BS<sup>35</sup>P

Ninety-eight and four-tenths per cent of this material migrated with the same  $R_f$  (0.75) as standard BSP. One and three-tenths per cent had an  $R_f$  of 0.50 and 0.3 per cent an  $R_f$  of 0.92.

dividing the optical density derived from that band by the sum of the optical densities of all the bands.

The distribution of BSP in different bands was also determined from segments of the entire chromatograph as follows: Strips 1 cm. wide were cut longitudinally from the chromatograms. The strips were then cut at 1 cm. intervals and the dye contained in each square cm. of paper was measured either colorimetrically or by counting when BS<sup>35</sup>P was used. For colorimetric analysis, the dye on each square was eluted into 4 ml. of distilled water for 30 minutes and the optical density of the eluate was determined after the addition of 0.1 ml. of 20 per cent potassium hydroxide. When radioactivity was assayed, each square was placed in a counting bottle to which 4 ml. of a 0.4 per cent solution of 2:5-diphenyloxazole in toluene was added, and the counts were measured in a liquid

scintillation counter. A linear counting response was obtained over the range measured in these studies, up to 50,000 counts per minute (cpm).

The optical densities and cpm of the BSP contained in each square were then plotted on the ordinate against the distance of the squares from the origin of the chromatograph in centimeters on the abscissa. Such a plot yielded a series of peaks corresponding to each BSP band. The per cent of BSP in each band was then calculated by dividing the sum of the optical densities or the counts contained in each peak by the sum of the optical densities or counts contained in all peaks. When the per cent distribution of BSP was obtained from colorimetric data, no correction was made for possible differences in the extinction coefficients of the various BSP compounds since values obtained from measurements of radioactivity and color on separate strips from a single chromatogram agreed closely (Table I). Despite the fact that the BSP content of only a segment of the chromatograph was measured, calculation of the per cent of BSP contained in each band from data obtained from individual strips did not differ appreciably from that obtained from measurements of the BSP content of the entire chromatograph (Table II). The quantity of BSP excreted in each band

TABLE I

*Comparison of the distribution of sulfobromophthalein sodium (BSP) compounds in bile from radioactivity and color*

Rat no.	Band	Counts	Color
		%	%
3	A	59.0	60.7
	B + C	17.7	14.2
	D	23.3	25.1
6	A	52.7	53.7
	B + C	25.3	22.9
	D	22.0	23.4
9	A	79.9	81.7
	B + C	4.7	3.2
	D	15.6	15.1
10	A	71.2	69.1
	B	9.0	8.9
	C	3.6	4.9
	D	16.1	17.1

TABLE II

*A comparison of the distribution of BSP in bile calculated from the radioactivity contained in strips and in an entire chromatograph*

Rat No. 5	Per cent of BSP contained in		
	Band A	Bands B and C	Band D
Strip 1	67.9	6.5	25.6
Strip 2	64.1	7.5	28.4
Strip 3	67.8	7.4	24.8
Entire chromatograph	66.7	7.2	26.1

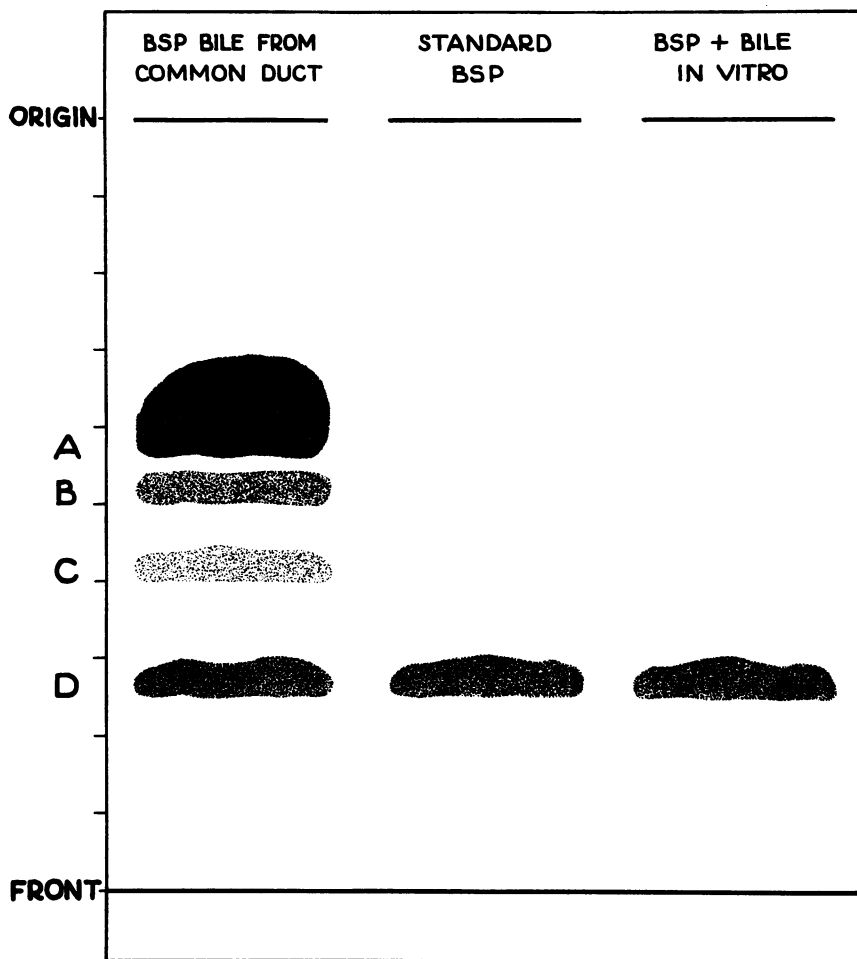


FIG. 2. DRAWING OF A DESCENDING CHROMATOGRAM OF BSP COMPOUNDS ON WHATMAN NO. 1 FILTER PAPER IN GLACIAL ACETIC ACID: WATER: *n*-PROPYL ALCOHOL (1: 5: 10, V/V)

was calculated from the values for the per cent of excreted BSP appearing in each band and the total amount of BSP recovered in bile.

#### RESULTS

##### 1) Chromatography of the biliary excretory products of BSP

Four and occasionally three distinct BSP bands were identified on chromatographs of bile obtained after BSP was administered intravenously. One of these, designated "D", migrated with the same  $R_f$  as standard BSP ( $R_f$  0.75), while Bands "A", "B" and "C" moved with average  $R_f$ 's of 0.44, 0.51 and 0.60, respectively (Figure 2). Separation of Bands B and C varied on different chromatographs. At times an excellent separation was obtained (Figure 2), whereas frequently these

bands migrated closely together (Figure 3). When BSP was incubated with control bile for as long as three hours *in vitro* and the mixture chromatographed, only a single band with the same  $R_f$  as standard BSP was observed. No evidence of a colorless BSP compound in bile was found after  $S^{35}$ -labeled BSP was administered and radioautographs of chromatograms were made (Figure 3).

##### 2) Distribution of BSP compounds in bile (Table III)

Eighty-two to 96 per cent of the injected BSP was recovered in the bile collected in a single tube for two and one-half to three hours following the intravenous administration of varying doses of BSP. An analysis of chromatograms of such bile

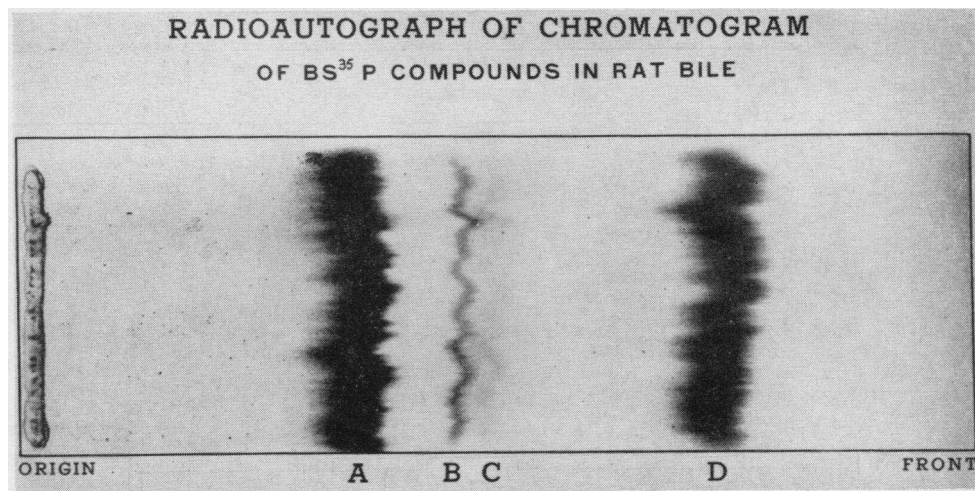


FIG. 3. RADIOAUTOGRAPH OF A CHROMATOGRAM OF BS<sup>35</sup>P COMPOUNDS IN BILE  
Four bands, A, B, C and D, each of which gave a purplish color on exposure to ammonia vapors, were identified. No colorless BSP compounds were detectable.

revealed that Band A contained the major part of the BSP, accounting for over 50 per cent of the dye excreted at the lowest dose administered to as much as 79.7 per cent when 2.62 mg. of BSP per 100 Gm. of body weight was administered. The quantity of BSP appearing in each band rose with increments in dosage, but Band A increased proportionally to a greater extent than Bands B, C and D (Figure 4).

### 3) Identification of BSP bands

The absorption spectrum of the compounds contained in the different bands is identical with that of standard BSP (Figure 5).

Ninhydrin reacting material is consistently associated with Bands A, B and C. The major compound comprising Band A has been subjected to the following analysis, which indicates that it is a conjugate of BSP with the amino acids, glycine and glutamic acid. When Band A was eluted from paper and subjected to acid hydrolysis (5.9 N HCl for three hours at 15 pounds pressure), it yielded bands corresponding to those of free BSP<sup>1</sup> and two ninhydrin spots. The latter were

<sup>1</sup> When standard BSP is subjected to acid hydrolysis and then chromatographed in acetic acid-water-*n*-propyl alcohol, two BSP bands can be identified. One migrates with the same  $R_f$  as standard BSP, the other moves

TABLE III  
Distribution of BSP recovered in rat bile after intravenous injection\*

Rat no.	BSP injected mg./100 Gm.	BSP excreted in bile (mg./100 Gm.)				Total	BSP excreted BSP injected
		Band A	Band B	Band C	Band D		
1	0.253	0.119		0.017†	0.088	0.224	0.885
2	0.257	0.111		0.042	0.067	0.220	0.856
3	0.478	0.259		0.078	0.101	0.438	0.924
4	0.482	0.233		0.035	0.172	0.440	0.912
5	0.987	0.596		0.064	0.233	0.893	0.904
6	1.096	0.527		0.254	0.221	1.002	0.914
7‡	1.640	1.170	0.212		0.196	1.578	0.962
8	1.700	1.212	0.102	0.068	0.173	1.555	0.914
9	2.620	1.840		0.109	0.369	2.315	0.883
10	2.650	1.542	0.195	0.078	0.349	2.170	0.818

\* Distribution in this table calculated from color in Rats 7 and 8 and from radioactivity in the others.

† The combined values for Bands B and C are listed when these bands could not be sharply separated from each other on the chromatograms for quantitative purposes. In such instances, however, both bands were identified.

‡ Bile collected for 150 minutes. Collection period was 180 minutes in other rats.

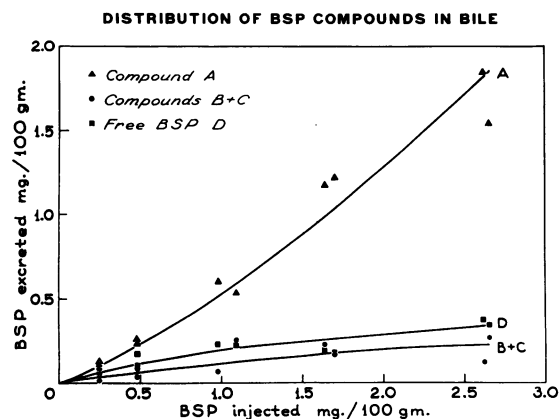


FIG. 4. THE DISTRIBUTION OF BSP COMPOUNDS EXCRETED INTO BILE PLOTTED AS A FUNCTION OF THE QUANTITY OF BSP INJECTED

identified as glycine and glutamic acid by the following: *a*) The ninhydrin spots assumed the positions of glycine and glutamic acid on two-dimensional chromatography in phenol-NH<sub>3</sub> and 80 per cent pyridine; *b*) when the unknown compounds were mixed with known samples of glycine and glutamic acid and chromatographed in the two-dimensional system, the unknown spots were reinforced; only two bands were seen, and these corresponded to the usual location of glycine and glutamic acid in this solvent system; *c*) the dinitrophenyl (DNP) derivatives of the unknown amino acids which were prepared moved with the same  $R_f$ 's as known DNP-glycine and DNP-glutamic acid in tertiary amyl alcohol-phthalate pH 6.0 buffer (15).

The possibility remained that the glycine and glutamic acid were not conjugated with BSP but appeared in bile either as free amino acids or as a dipeptide that migrated to the same position as Band A. Indeed, when control bile is chromatographed, ninhydrin reacting material with the same  $R_f$  as the BSP band may be identified in some specimens. However, this material can be removed by eluting it from paper and passing it through a Dowex 50- $\times$ 8 200-400 mesh ion exchange column. Compound A is not affected by this procedure.

A visual comparison of the size of the spots and the intensity of the ninhydrin color developed faster. These two bands are also found after compound A is hydrolyzed.

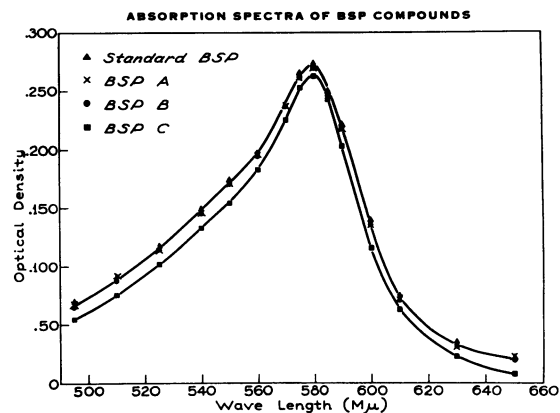


FIG. 5. ABSORPTION SPECTRA OF BSP COMPOUNDS EXCRETED INTO BILE

by glycine and glutamic acid derived from the BSP conjugate with known standards suggests that these amino acids are present in equimolar concentrations and that for each mole of BSP there is a mole of glycine and of glutamic acid. No evidence for free or combined -SH groups was found since negative nitroprusside reactions were obtained on eluates of conjugate A whether previously treated with NaCN or not; and specifically, there was no sign of cysteine, taurine or methionine on amino acid chromatograms.

No evidence of a BSP-glucuronic acid conjugate was found. Thus, chromatograms containing the same volume of control bile and bile containing BSP were developed. Band A and a comparable area from the control bile chromatogram were cut out and their contents were eluted into distilled water. The quantity of hexuronic acid (16) in the eluate of compound A did not differ significantly from that contained in control bile migrating with the same  $R_f$  as A, and was much less

TABLE IV  
Quantity of glucuronic acid associated with Band A

Rat	Quantity of BSP eluted from Band A	Glucuronic acid	
		Theoretical content if A were a monoglucuronide of BSP	Found in excess of that in control bile
no.	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$
4-14	300	67.1	<0.5
6-21	156	35.0	2.9
7-1	316	70.9	<0.5

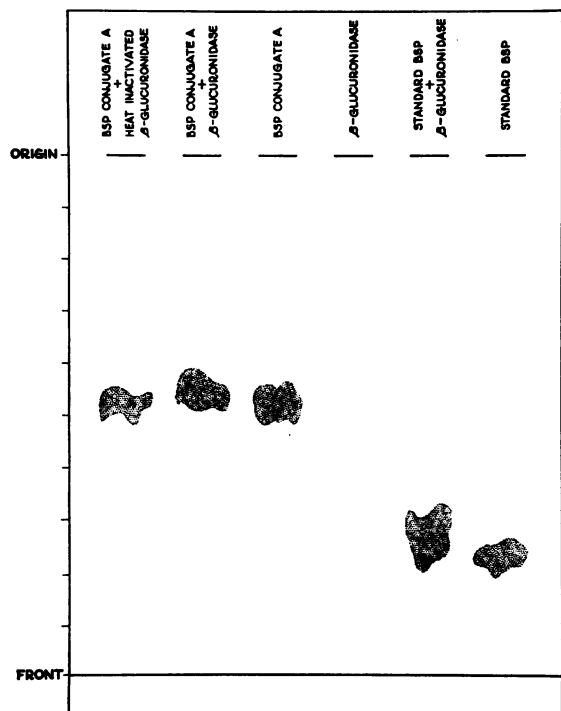


FIG. 6. DRAWING OF A DESCENDING CHROMATOGRAM OF STANDARD BSP AND CONJUGATE A AFTER INCUBATION WITH  $\beta$ -GLUCURONIDASE

No free BSP is liberated from A and the  $R_f$  of A is not altered.

than that amount which would have been present if A were a monoglucuronide of BSP (Table IV).

In addition, no free BSP was detected by paper chromatography when compound A was incubated with  $\beta$ -glucuronidase (Worthington) at 37°C., pH 4.9, for one hour, nor was the  $R_f$  of A altered (Figure 6). Under similar conditions, the activity of the enzyme was demonstrated by its capacity to liberate phenolphthalein from phenolphthalein glucuronide.

#### DISCUSSION

The primary purpose of the present report is to describe the nature of the major biliary excretory product of BSP in the rat. This compound, designated BSP A, accounts for up to 80 per cent of the BSP recovered in bile. Ninhydrin reacting material has been found to conform both in shape and position with BSP A on paper chromatograms. This ninhydrin moiety has been demonstrated to be an integral part of the com-

pound since it cannot be removed by passing BSP A through a Dowex 50- $\times$ 8 ion exchange resin. When subjected to acid hydrolysis, BSP A yields free BSP and two ninhydrin spots. The latter have been identified as the amino acids, glycine and glutamic acid, by means of two-dimensional filter paper chromatography, and this has been confirmed by identifying the dinitrophenyl derivatives of these amino acids. Thus, the major biochemical process concerned with the excretion of BSP into bile is one of conjugation of BSP with glycine and glutamic acid. Ninhydrin reacting material is also associated with BSP B and BSP C, indicating that conjugation with amino acids is also involved in the excretion of these compounds. Studies concerned with the identification of these amino acids are currently in progress.

A preliminary analysis of BSP compounds in human T-tube bile (17) also reveals that the major excretory product is intimately associated with ninhydrin positive material. As in the rat, glycine and glutamic acid are recovered after acid hydrolysis and alanine has been observed in some specimens. These findings in the rat and the preliminary observations in man suggest, therefore, that the BSP test is a measure, at least in part, of amide formation in the liver.

No evidence for a BSP-glucuronic acid conjugate has been found in the present studies in the rat and in man (17). Krebs and Brauer also did not identify a BSP glucuronide compound in rat bile (18). These observations are consistent with the clinical finding that BSP removal by the liver is normal in patients with Gilbert's disease (19), a disorder characterized by impairment of glucuronide conjugation in the liver (20, 21).

The present studies do not exclude the possibility that the BSP molecule itself is altered during excretion by the liver. The absorption spectra of the BSP compounds appearing in rat bile were identical, however, suggesting that the BSP molecule remains intact. Certainly no colorless BSP compounds in bile were found when radioautographs of chromatograms were made after  $S^{35}$ -labeled BSP was administered.

Recently, Krebs and Brauer have reported on their separation of BSP products in bile of a variety of animal species by means of chromato-

graphy on alumina columns (18). In the rat, they identified three BSP compounds which they designated "I," "II" and "III." Despite differences in the methods used for separation of BSP compounds in their studies and in the present report, a similarity of the BSP products is apparent. Thus, Compound I was identified as free BSP and is probably the same as BSP D. Compound III accounted for most of the BSP excreted in rat bile. Although its biochemical nature was not defined, it seems likely that it is identical with BSP A. Compound II may be the same as BSP B or BSP C or conceivably contains a mixture of both B and C.

Evidence has been presented (5-14) which indicates that the hepatic removal of BSP depends upon the simultaneous operation of at least two processes: 1) uptake by liver cells into a "storage compartment" in an amount proportional to plasma concentration, and 2) removal from blood by a transfer mechanism characterized by a transfer maximum or  $T_m$ . It has been inferred that the  $T_m$  is a measure of the movement of BSP from blood to bile (13, 14). The finding of four distinct BSP compounds in bile in the present studies introduces a new complexity in the interpretation of the values for  $T_m$ . Henceforth, any analysis of BSP  $T_m$  must take into consideration the probability that more than one transport process is operative in the movement of BSP into bile.

#### SUMMARY

An analysis of sulfobromophthalein sodium (BSP) compounds appearing in the bile of the rat has been undertaken. Usually four and occasionally three BSP bands have been identified by paper chromatography. The major excretory product of BSP in bile, designated BSP A, accounts for up to 80 per cent of the recovered dye. This compound and its hydrolytic products were subjected to both chromatographic and chemical analysis, the results of which indicate that BSP is excreted by the liver of the rat primarily as a conjugate of glycine and glutamic acid.

#### ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. Marvin D. Siperstein for many helpful suggestions and to Mrs. Mary Hamilton and Mrs. Sue Stakelum for valuable technical assistance.

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