ing protein synthesis or direct stimulation of beta cells to anatomical and functional hypertrophy(14).

Summary. Hypoglycemic responses of normal and diabetic men to tolbutamide and indole-3-acetic acid were similar both qualitatively and quantitatively. These substances, respectively, depressed fasting glucose levels 22% and 11% in normal subjects; 44% and 35% in elderly patients with mild diabetes; and 1% and 7% in uncontrolled juvenile diabetics. Normal individuals and mild diabetics demonstrated significant "insulin activity" in their fasting plasmas, and both groups exhibited sharp increases in insulin activity after ingestion of glucose. Insulin activity was not detectable in blood of juvenile diabetics either before or after glucose. In no individual, however, whether normal or diabetic, was the hypoglycemic response to tolbutamide or indole-3-acetic acid accompanied by increased "plasma insulin activity" in peripheral blood.

1. Dulin, W. E., Johnston, R. L., *Ann. N. Y. Acad. Sci.*, 1957, v71, 177.

2. Mirsky, I. A., Diengott, D., Perisutti, G., *Endocrinology*, 1956, v59, 715.

3. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

4. Vallance-Owen, J., Hurlock, B., *Lancet*, 1954, v1, 68.

5. Mirsky, I. A., Diengott, D., PROC. SOC. EXP. BIOL. MED., 1956, v93, 109.

6. Vallance-Owen, J., Hurlock, B., Please, N. W., *Lancet*, 1955, v2, 583.

7. Ricketts, H. T., Wildberger, H. L., Schmid, H., *Ann. N. Y. Acad. Sci.*, 1957, v71, 170.

8. Pfeiffer, E. F., *Abstr. Am. Chem. Soc. Meet.*, 1958, (April), 17M.

9. Madison, L. L., Kaplan, N., *J. Lab. Clin. Med.*, 1958, v52, 927.

10. Bearn, A. G., Billing, B. H., Sherlock, S., *Clin. Sci.*, 1952, v11, 151.

11. Recant, L., Fischer, G. L., *Ann. N. Y. Acad. Sci.*, 1957, v71, 62.

12. Renold, A. E., Martin, D. B., Boshell, B. R., Thorn, G. W., *ibid.*, 1957, v71, 71.

13. Metz, R., *J. Lab. Clin. Med.*, 1958, v52, 929.

14. Batts, A. A., Bennett, L. L., Ellis, S., George, R. A., *Endocrinology*, 1956, v59, 620.

Received September 4, 1958. P.S.E.B.M., 1959, v100.

Metabolism of Sulfobromophthalein Sodium (BSP) in Dog and Man.*† (24563)

JAY I. MELTZER, HENRY O. WHEELER,‡ AND WILLIAM I. CRANSTON§
(Introduced by Stanley E. Bradley)
Dept. of Medicine, Columbia University, College of Physicians and Surgeons, and Presbyterian Hospital, N. Y. City

Incomplete recovery of sulfobromophthalein sodium (BSP) from bile following intravenous administration has been attributed either to extrahepatic removal(1) or to BSP metabolism by the liver(2). Brauer and his associates(3,4,5) clarified this problem by a variety of technics including column chromatographic analysis with S^{35} labelled dye as tracer. They have shown that BSP is altered by liver and that the products in bile are colored compounds resembling BSP in their absorption spectra, but apparently differing with respect to extinction coefficients. As a result, colorimetric determinations of BSP in bile are affected by an error leading to systematic underestimation of concentration. Recent studies(6,7) indicate that BSP is transferred into bile by a rate-limited transport system and that it accumulates in hepatic cells in some fixed concentration gradient with respect to plasma. Further understanding of these

* This work was made possible by grants from N. Y. Heart Assn. and Dept. of Army.

† The authors are indebted to Mrs. Katharine Baker for assistance in identification of amino acids, and to Misses Marion Yulinsky and Evelyn Audioun and Mrs. Susan Stassa for technical help.

‡ John and Mary R. Markle Scholar in Medical Science.

§ Rockefeller Travelling Fellow in Medicine.

functions requires a knowledge of the character and behavior of the metabolites of BSP. We approached this problem by utilizing the technic of paper chromatography on specimens of plasma and bile obtained during intravenous BSP infusion.

Methods. Four cholecystectomized, splenectomized dogs were fitted with a Thomas duodenal fistula apparatus(8), placed so that the Ampulla of Vater could be visualized. After inserting a ureteral catheter directly into the common bile duct, fasting unanesthetized animals were maintained in upright position by a sling. This preparation permitted accurate collection of timed bile samples by aspiration or gravity drainage. Venous blood was obtained from an indwelling polyethylene tubing. In 5 human subjects, 2 post-cholecystectomy, one with active infectious hepatitis and 2 normal, blood was obtained by venipuncture, and in the first 2 of these, bile was collected from a T-tube. Each of 4 dogs and 5 human subjects was given intravenous infusion of BSP by means of constant delivery pump at a rate equal to or exceeding maximal excretory rate or Tm_{BSP}, which was measured (7). After suitable equilibration period appropriate blood and bile specimens were taken and all analyzed as follows for metabolites of BSP: *1. Preparation of samples for chromatography.* (a) *Plasma concentrations* of BSP were determined in Beckman DU spectrophotometer set at 580 mμ after dilution and addition of alkali. BSP was extracted into protein-free solution and concentrated as follows: Two ml of plasma were mixed thoroughly with 0.36 ml of a saturated solution of ammonium sulfate, shaken with 5 ml of ethanol and maintained at room temperature 30 minutes. After centrifuging, the clear supernatant was aspirated and transferred to a clean tube. In 28 determinations 94.3 ± (S.D.) 4.5% of BSP in original plasma sample was recovered. The supernatant was then evaporated to dryness under air stream in water bath at 40 to 50°C. BSP in the residue was extracted into 0.3 ml of ethanol prior to chromatography. (b) *Biliary concentrations* of BSP, measured colorimetrically, ranged 1000 to 1500 mg %. The high concentrations permitted direct analysis, without concentration or extraction. Occasionally high concentrations of various interfering substances necessitated precipitation and extraction of BSP from bile by the method employed for plasma. Repeated ethanol extractions were required in the final step to ensure complete recovery. No significant differences were observed between chromatograms of extracted and unextracted bile samples when the latter were technically adequate. *2. Chromatography.* The samples were applied in duplicate on Whatman #1 filter paper (17 x 21 inches) in successive volumes of 10 λ or less, allowing each to dry before reapplying until approximately .01 to .02 mg of BSP had accrued. Solvent was prepared as follows: To 4 parts of reagent grade n-butanol mixed with one part of glacial acetic acid distilled water was added in excess. The edge of the prepared filter paper was dipped in the solvent in a sealed container and allowed to stand while the solvent ascended 25-30 cm, usually 14-16 hours at 20°C. The paper was then dried and "developed" by spraying each side with 20% sodium carbonate. *3. Quantitative methods.* Chromatograms revealed one or more discrete areas occupied by BSP-like compounds. Each area (spot), assigned a separate R_f, was cut out and the dye eluted from the paper into 5 ml of distilled water plus 0.1 ml of 30% potassium hydroxide. The amount of BSP in each was calculated from the optical density of its eluate minus an appropriate blank, using a curve calibrated with BSP. The sum of optical densities of all components of the chromatogram gave average recovery of 86% of total applied at starting point, in 9 determinations. The percentage of each BSP-like compound in a given sample was expressed in terms of optical density of its eluate to the sum of optical densities of all components. This percentage includes a systematic error depending upon the extent of assumed but as yet undetermined differences between extinction coefficients of BSP and its metabolites in dog and man. Analysis of chromatograms by this method revealed agreement within 5% in duplicate determinations of any one component in 96 instances.

TABLE I. Average R_f Values of BSP-Like Compounds Found in Bile and Plasma during Constant Intravenous Infusions of BSP.

Subject	Sample	Chromatographic components			
		BSP-I	BSP-II	BSP-III	BSP-IV
Dog	Bile	.48 (52)	.38 (51)	.34 (50)	.30 (50)
Man	"	.47 (2)	.33 (2)	.30 (2)	.28 (2)
"	Plasma	.47 (21)	.38 (9)	.32 (17)	.27 (17)

No. in parentheses indicate No. of determinations.

Results. 1. Demonstration of BSP metabolites in bile and plasma. Four compounds whose absorption spectra were identical with BSP were demonstrated chromatographically in all bile samples of 4 dog and 2 human subjects, and in plasma of 5 human subjects. A photograph of a typical chromatographic pattern of dog bile is shown in Fig. 1. Table I shows average R_f values of the various compounds in the chromatograms, referred to in descending order as BSP-I, BSP-II, BSP-III and BSP-IV. (Chromatograms of dog plasma, although showing presence of several BSP compounds, were technically unsatisfactory). These patterns appear to be similar to those described by Krebs and Brauer(5) for bile of sheep, cat, dog, chicken and rat subjected to column chromatographic analysis. Since similarity of pattern does not necessarily imply chemical identity of the metabolites between species, the animal source should be indicated. The major compound (97.3%) in commercially available BSP was chromatographically identical with BSP-I in the bile and plasma samples. In 2 lots of commercial dye tested, 2 impurities with BSP color were identified chromatographically at R_f 0.95 (0.5%) and 0.84 (2.2%), referred to respectively as BSP-a and BSP-b. The chromatographic character of BSP was unaffected by incubation at 37°C for from 3 to 12 hours with plasma, whole blood and bile from dogs and from normal and jaundiced human subjects. It is therefore apparent that formation of BSP-II, III and IV is dependent upon a metabolic process *in vivo*.

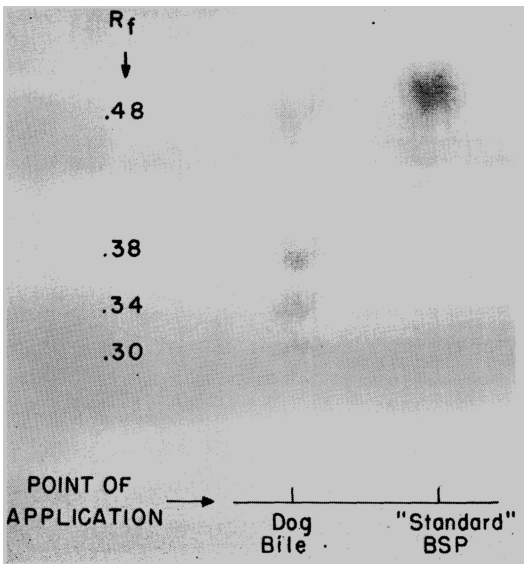

FIG. 1. Photograph of a chromatogram of BSP as excreted in dog bile (left) and as pure BSP (right). The 4 chromatographic components are clearly separated, referred to in descending order as BSP-I, II, III and IV.

2. Properties of chromatographic components. (a) BSP-I was hydrolyzed in 6N hydrochloric acid for 18 hours at 110°C in sealed tubes. Seventy-five per cent of total color was recovered. Chromatography following this treatment revealed that approximately half the dye was unaffected and the remainder consisted of BSP-a and BSP-b. *(b) BSP metabolites.* Insofar as identity of R_f suggests chemical identity, the corresponding compounds from different dogs appear to be the same. This was confirmed by the fact that mixtures of bile samples from different dogs yielded chromatograms identical in pattern. The 2 human samples were not mixed because they were obtained weeks apart. Each of the metabolites in dog bile was chromatographically stable to boiling in air for 3 hours, to addition of 6N HCl, to addition of alkali to pH 12, to drying, and to storage at 4°C. The reduced R_f values of metabolized BSP suggested the possibility of conjugation with one or more polar groups. Hydrolysis with 3N hydrochloric acid for 3 hours at 100°C to split possible glucuronic linkages

TABLE II. Percentage Distribution of BSP and Metabolites in Dog Bile Excreted during an Intravenous Infusion Equal to Tm_{BSP}.

Time, min.	Blood level BSP, mg/100 ml	Bile flow, ml/min.	Total colorimetric BSP output-bile, mg/min.	% of total color in bile of BSP and metabolites			
				I	II	III	IV
22	3.42	.188	2.97	13	54	8	24
39	3.23	.209	2.94	13	55	10	22
50	3.25	.204	2.97	14	55	10	22
68	3.52	.185	2.51	13	55	11	20

did not alter chromatographic mobility of the metabolites. All 3 metabolites in dog bile and BSP-III in human bile in contrast to commercially available BSP or BSP-I in bile, were found to be linked with a ninhydrin reactive substance.

The question of chemical identity of corresponding metabolites of dog and man was not fully answered in this study. However, in contrast to their counterparts in the dog, BSP-II and BSP-IV in human bile were not shown to be linked to a ninhydrin reacting compound. The major constituents of dog and human bile, BSP-II and BSP-III respectively, do not seem to correspond. Present technics permitted isolation of only major fractions in bile and these 2 were analyzed further. BSP-II-dog and BSP-III-man were hydrolyzed with 6N hydrochloric acid in sealed tubes for 18 hours at 110°C. The products of hydrolysis were subjected to 2-dimensional chromatography for amino acids (9,10). Canine BSP-II yielded a single amino acid identified as glycine. Human BSP-III yielded at least 3 amino acids identified as alanine, glycine and glutamic acid. Cysteine and cystine were not detected. Both hydrolysates also contained 2 new derivatives of BSP which moved at R_f .60 and .66 in the n-butanol solvent. These compounds have an absorption spectrum similar to BSP and were not detected in the hydrolysis of BSP-I. Small amounts of BSP-I were also found in the hydrolysates.

3. Physiologic properties of BSP metabolites. A constant biliary excretory rate was observed when infusions of BSP were administered to dogs at rates in excess of Tm_{BSP} (7). Table II shows the proportion of total BSP color attributable to the 4 fractions excreted in bile of dog Cora at various times during constant infusion equal to her previously measured Tm_{BSP}. Table III lists average proportions of the 4 fractions during sustained infusion at or above the excretory maximum on 5 occasions in 4 dogs. The data indicate that BSP and its metabolites are excreted in constant proportions in a given animal and in very nearly the same proportions from dog to dog under conditions of maximal biliary excretory rate of BSP. This did not seem to be the case prior to stabilization at Tm levels. Metabolites could not be detected in plasma of dog or man until at least 30 minutes had elapsed following start of a constant infusion of BSP. The presence of metabolites

TABLE III. Comparison of Mean Percentage Distribution of BSP and Metabolites in Bile of 4 Dogs Obtained during Constant Intravenous Infusion of BSP at Rates Equal to or in Excess of Tm_{BSP}.

Dog	Bile flow, ml/min.	Total colorimetric BSP output-bile, mg/min.	% of total color in bile of BSP and metabolites				No. of determinations
			I	II	III	IV	
Cora	.20	2.8	13	55	10	22	4
Norma	.32	3.8	16	59	16	10	3
"	.31	3.4	11	63	16	11	2
Isolde	.32	3.7	10	53	17	21	4
Tosca	.27	2.8	13	58	23	7	3

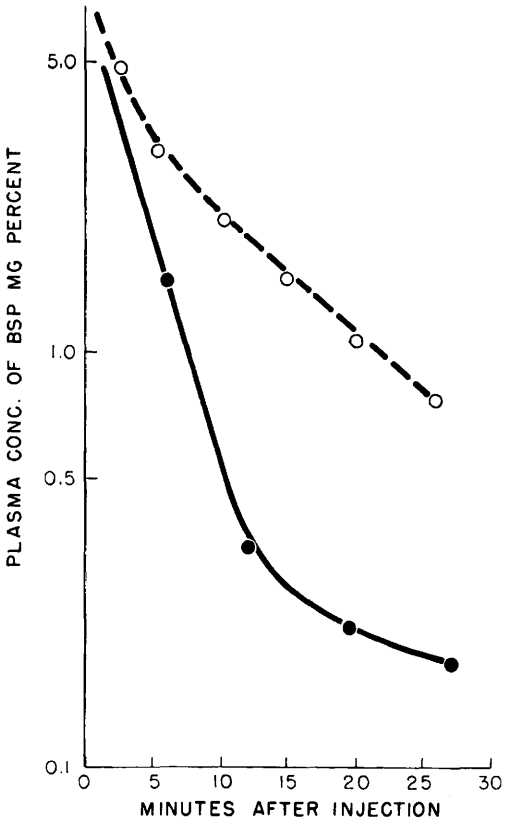

FIG. 2. Comparison of disappearance from plasma following a single intrav. inj. of a mixture of BSP metabolites (○- - -○) and following administration of same amount of unaltered BSP (●——●) to same dog on different days. Each dose approximately 50 mg.

was most evident in plasma of human subjects with impaired liver function. A solution of metabolites alone was prepared by elution of appropriate areas of a bile chromatogram. This was passed through a Seitz filter and given intravenously to dog Cora. Fig. 2 compares disappearance from plasma of this mixture of BSP-II, III and IV, and a similar amount of unaltered BSP. The normal dog removed unaltered BSP more rapidly than its metabolites following a single intravenous injection. As measured colorimetrically, 97.4% of injected metabolites was recovered in bile in 3 hours, suggesting that the lower recovery of commercial BSP may be due to differences in extinction coefficients(5). Metabolites were not excreted in the same proportion as in the "injectate." Trace amounts of BSP-I, not present in injected material, were found in the last bile samples collected. There also appeared to be some conversion of BSP-II to BSP-III.

Discussion. This study confirms the observations of others that BSP is metabolized by dog and man(5,11). Our evidence supports the conclusion(5,11) that metabolites are not glucuronides. Part of the metabolic change appears to involve conjugation with one or more amino acids. That this is not the only alteration in the molecule is suggested by the following observations: 1) There are significant differences with respect to presence of a ninhydrin reacting moiety in metabolites in dog and human bile although the corresponding metabolites have approximately the same R_f values, 2) 6N acid hydrolysis of 2 metabolites liberate a new BSP derivative, whereas a simple conjugation would be expected to yield unaltered BSP.

Since metabolites are formed by the isolated perfused rat liver(12), and the liver is the major site of BSP removal from plasma, the liver is probably the site of formation of BSP metabolites in dog and man. The presence of these compounds in plasma as well as bile raises the question of whether other tissues may also be capable of metabolism of BSP. Analysis of plasma of hepatectomized animals during BSP infusion will be necessary to answer this question. Our finding that metabolites appear in bile of dogs prior to their detection in plasma is compatible with the inference that the source of plasma components is the liver. The high recovery in bile of intravenously injected metabolites demonstrates that the liver removes these substances efficiently, although at a slower rate than unaltered BSP. Reinjected metabolites also appear to be altered slightly in the process of reexcretion. These findings indicate that removal and excretion of infused BSP from plasma involve the possibility of a number of reactions which proceed simultaneously. The magnitude of these various reactions has yet to be assessed.

At maximal transfer rates quantitatively consistent distribution of BSP metabolites was observed in bile. The significance of this find-

ing is uncertain. It may mean that an excretion maximum exists for each metabolite, but further study is necessary to test this hypothesis. It is apparent from the data that calculation of a true BSP transport maximum is feasible from measurement of total color, percentage distribution of chromatographic fractions and their extinction coefficients.

Summary. During administration of a constant infusion of BSP in the dog and man, 3 chromatographically distinct metabolites were demonstrated in plasma and bile in addition to BSP itself. Part of the metabolic change involves conjugation of BSP or some BSP derivative with one or more amino acids. Some of the physiologic properties of BSP metabolites are described and discussed, particularly with reference to problems of BSP transport from plasma to bile.

1. Brauer, R. W., Pessotti, R. L., *Am. J. Physiol.*, 1950, v162, 565.

2. Wirts, C. W., Cantarow, A., *Am. J. Digest. Dis.*, 1942, v9, 101.

3. Brauer, R. W., Krebs, J. S., Pessotti, R. L., *Fed. Proc.*, 1950, v9, 259.

4. Brauer, R. W., Pessotti, R. L. Krebs, J. S., *J. Clin. Invest.*, 1955, v34, 35.

5. Krebs, J. S., Brauer, R. W., *Am. J. Physiol.*, 1958, v194, 37.

6. Combes, B., Wheeler, H. O., Childs, A. W., Bradley, S. E., *Trans. Assn. Am. Phys.*, 1956, v64, 276.

7. Wheeler, H. O., Meltzer, J. I., Epstein, R. M., Bradley, S. E., *J. Clin. Invest.*, 1958, v37, 942.

8. Thomas, J. E., PROC. SOC. EXP. BIOL. AND MED., 1941, v46, 260.

9. Datta, S. P., Dent, C. E., Harris, H., *Science*, 1950, v112, 621.

10. Lathem, W., Baker, K., Bradley, S. E., *Am. J. Med.*, 1955, v18, 249.

11. Grodsky, G. M., Fansha, R., Carbone, R. V., *Physiol.*, 1958, v1, 29.

12. Brauer, R. W., Pessotti, R. L., Pizzolato, P., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 124.

Received October 2, 1958. P.S.E.B.M., 1959, v100.

Feather Depigmentation Resulting from Feeding Molybdenum Plus Thiosulfate.* (24564)

E. C. MILLER AND C. A. DENTON

U. S. Dept. of Agriculture, Animal Husbandry Research Division, Beltsville, Md.

Achromatosis of feathers has been observed in chicks due to deficiency of lysine(1), pantothenic acid(2), and folic acid(3). Achromatosis of the hair of rats resulting from copper deficiency could be corrected by additional dietary calcium pantothenate(4). Although excess dietary molybdenum caused achromatosis of hair in cattle, the literature did not reveal reports of feather depigmentation in chickens due to excess dietary molybdenum. In studies of Miller *et al.*(5) on effect of sulfur containing compounds in alleviation of a molybdenum-induced growth inhibition, it was observed that addition of sodium thiosulfate to a diet containing 1500 ppm molybdenum resulted in feather depigmentation. The data presented here show that feather depigmentation resulting from feeding molybdenum and thiosulfate can be prevented by addition of copper to the diet.

Methods. One-week-old New Hampshire X Barred Plymouth Rock female chicks (10/group) were used except for experiment in which sodium thiosulfate-S^{35} was fed to day-old chicks. The chicks were housed in electrically heated brooders with raised wire floors, and fed the experimental diet for 3 weeks. Feed and water were supplied *ad lib.* Sodium molybdate dihydrate and sodium thiosulfate pentahydrate supplemented the basal diet (Table I). Molybdenum and copper content of basal diet and liver tissues of chicks was determined. The method of Evans *et al.*(6) was used for molybdenum determination and that of Andrus(7) for copper determination. Bone ash was determined by

* This project was supported in part by U. S. Atomic Energy Comm.