than they did under the treatments that had periods of no indicator movement. The small numerical differences between treatments and the large variability between subjects restrict the practical implications of these findings. Of greater significance is the similarity of these data to the results of certain experiments with other animals (*4*). Taken altogether, these studies suggest that many more conditions can sustain or even form habits than have traditionally been acknowledged by psychologists.

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Biliary Excretion by the Rat of Bromsulfalein as a Conjugate of Glycine and Glutamic Acid

Abstract. Paper chromatograms of bile collected after the intravenous administration of bromsulfalein to rats reveal four distinct bromsulfalein bands. One migrates with the same R_f as standard bromsulfalein; the other three move less fast. The major band, which accounts for 71.3 percent of the injected bromsulfalein, appears to be a conjugate of bromsulfalein with glycine and glutamic acid.

Bromsulfalein (sodium phenoltetrabromphthalein disulfonate) (BSP) is one of a group of phthalein dyes that is removed from blood predominantly by the liver and excreted into the bile (*1*). Dye removal is impaired in the presence of hepatocellular damage, and BSP retention in blood has proved to be a sensitive index of hepatic dysfunction (*2*). Although this dye has been used extensively in the clinical detection of hepatic disease, the precise mechanisms by which it is handled by the liver are still poorly understood.

A considerable body of evidence (*3, 4*) suggests that hepatic removal of BSP depends upon the simultaneous operation of at least two processes: (i) uptake of dye by liver cells until the cellular space is saturated with respect to a given blood level, and (ii) transfer from blood to bile by a rate-limited transfer mechanism. In order to further examine the process of biliary excretion, an analysis of BSP as it appeared in the bile of the rat was undertaken (*5*).

Fine polyethylene tubing was inserted into the common bile ducts of rats (Long-Evans, Wistar) under ether anesthesia, and 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, approximately 5 mg of BSP was injected intravenously, and additional bile samples were obtained. Aliquots of the bile were applied to Whatman No. 1 filter paper, and the chromatograms were developed in a descending system consisting of glacial acetic acid:water:*n*-propyl alcohol (1:5:10 vol./vol.). Usually four and occasionally three chromatographically distinct BSP bands were identified in bile by (i) the development of a purplish color on exposure of the paper to ammonia vapors, and (ii) absorption in the ultraviolet (Fig. 1). One of these bands, band D, migrated with the same R_f (0.75) as standard BSP, while bands A, B, and C moved less far, with average R_f's of 0.44, 0.51, and 0.60, respectively. When BSP was incubated with control bile for as long as 3 hours in vitro and the mixture was chromatographed, only a single band with the same R_f as standard BSP was observed (Fig. 1).

The distribution of BSP between the different bands in bile obtained from two rats is presented in Table 1. Bile was collected in a single tube for 150 and 180 minutes, respectively, after the intravenous administration of BSP, and aliquots were chromatographed; the bands were identified, cut out, and eluted with water; and BSP content was determined colorimetrically after addition of 20-percent KOH to appropriately diluted samples. It is apparent that band A contained most of the excreted BSP, accounting for 71.2 and 71.4 percent, respectively, of the total BSP injected in these two rats. Bands B and D contained smaller amounts of BSP. Band C was identified in one of these specimens, and when observed in bile from other rats it contained only very small quantities of BSP, as judged from the intensity of the purple color which developed on exposure of the paper to ammonia.

Table 1. Distribution of BSP recovered in bile after intravenous injection.

Amt. injected (mg)	Amt. recovered in bile (% of amt. injected)				
	Band				Total
	A	B	C	D	
*Rat No. 6-21** (wt. 312 g)					
5.13	71.2	12.8		11.9	95.9
Rat No. 7-1† (wt. 342 g)					
5.90	71.4	6.0	4.0	10.2	91.6

* Bile collected for 150 minutes.
† Bile collected for 180 minutes.

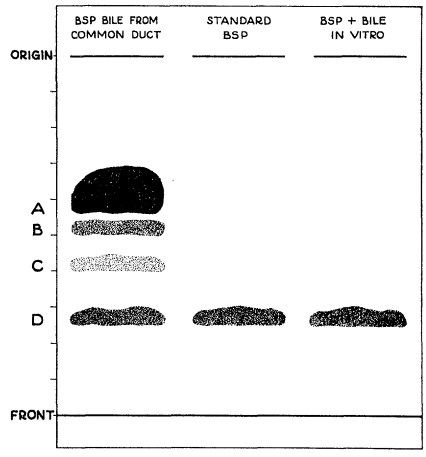

Fig. 1. Descending chromatogram on Whatman No. 1 filter paper in glacial acetic acid:water:*n*-propyl alcohol (1:5:10, vol./vol.).

The compound comprising band A has been subjected to further analysis, which indicates that it is a conjugate of BSP with the amino acids glycine and glutamic acid. Ninhydrin-reacting material which conformed both in shape and position to the ammonia reaction of band A was seen on chromatograms developed in the one-dimensional descending system (acetic acid:water:*n*-propyl alcohol) and a two-dimensional ascending (phenol-NH_3 and 80-percent pyridine) system. When band A was eluted from paper and subjected to acid hydrolysis in 5.9*N* HCl for 3 hours at 15 lb pressure, it yielded bands corresponding to those of free BSP (*6*), and two Ninhydrin spots. The latter were identified as glycine and glutamic acid from the following observations: (i) the Ninhydrin spots assumed the positions of glycine and glutamic acid on two-dimensional chromatography in phenol-NH_3 and 80-percent pyridine; (ii) when the unknown compounds were mixed with known samples of glycine and glutamic acid and chromatographed in the two-dimensional system, only two bands were seen, and these corresponded to the usual location of glycine and glutamic acid standards in this solvent system; (iii) the dinitrophenyl (DNP) derivatives of the unknown amino acids moved with the same R_f's as known DNP-glycine and DNP-glutamic acid in tertiary amyl alcohol-phthalate buffer (*p*H 6.0) (*7*).

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 speci-

mens. However, this material can be removed by eluting it from paper and passing it through a Dowex 50 – × 8 200-400 mesh column. Compound A is not affected by this procedure.

A visual comparison with known standards of the size of the spots and the intensity of the Ninhydrin color developed by glycine and glutamic acid derived from the BSP conjugate suggests that these amino acids are present in equimolar concentration and that for each mole of BSP there is a mole of glycine and a mole of glutamic acid. Chemical analyses to confirm this impression are now in progress.

Tests on band A for free or combined sulfhydryl groups were negative. It should be mentioned that no evidence of a BSP-glucuronic acid conjugate was found. Thus, the quantity of hexuronic acid (*8*) in compound A did not differ significantly from that contained in control bile migrating with the same R_f as A, and after incubation of A with β-glucuronidase (Worthington) at 37°C, *p*H 4.9, for 1 hour, no free BSP was detected by paper chromatography. Under similar conditions, the activity of the enzyme was demonstrated by its capacity to liberate phenolphthalein from phenolphthalein glucuronide.

The results of this study indicate that BSP is excreted in the bile of the rat as at least three and perhaps four compounds. This finding introduces a new complexity in the interpretation of the values for BSP T_m obtained in previous investigations (*4, 9*). Any analysis of biliary secretory T_m of BSP must henceforth take into account the probability that more than one transport process is operative in the movement of BSP from blood to bile.

The primary purpose of this communication is to describe the nature of the major excretory product of BSP in bile. This compound and its hydrolytic products were subjected to both chromatographic and chemical analysis. The results of these studies indicate that BSP is excreted by the liver of the rat primarily as a conjugate of glycine and glutamic acid, over 75 percent of the excreted BSP being found in this compound.

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6. Standard BSP, when subjected to acid hydrolysis and then chromatographed in the acetic acid:water:*n*-propyl alcohol solvent system, yields two BSP bands, one migrating with the same R_f as BSP, the other moving faster. These two bands are also identified after A is hydrolyzed.
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An Artificial Reagent for the Diagnosis of Classical Hemophilia

Present methods for the laboratory diagnosis of classical hemophilia [hemophilia A, or antihemophilic globulin (AHG) deficiency] are modifications of the thromboplastin generation test (*1*) or are dependent upon the use of plasma from a patient with classical hemophilia as reagent (*2*). The first of these methods, although of confirmed value, is time-consuming; a disadvantage of the second method is that adequate amounts of hemophilic blood are not always easily available. For these reasons an alternative rapid method in which readily available materials are used would be of value.

The principle of an alternative method was suggested by the observations (*3*) of the effects of disodium ethylenediaminetetracetic acid (Na_2-EDTA) on certain clotting factors; rapid and pronounced losses of proaccelerin (factor V) and AHG have been the most marked findings (*4*).

Normal human blood was collected into Na_2-EDTA (*5*) (150 mg/100 ml of blood). After centrifugation the plasma was decanted and stored at +4°C for 30 days. Plasma so treated retained normal activities of plasma thromboplastin component (PTC), plasma thromboplastin antecedent (PTA), Hageman factor, prothrombin, proconvertin [factor VII, or serum prothrombin conversion accelerator (SPCA)], Stuart factor, and fibrinogen; however, AHG and proaccelerin fell to undetectable levels (< 0.1 percent). Such plasma was stored at −20°C in small aliquots until needed. Optimal quantities of Asolectin (*6*) and barium sulfate adsorbed oxalated bovine serum were added to such plasma immediately before the test, as sources of platelet prothromboplastic-factor–like activity and of accelerin, respectively. The buffer for all dilutions was Veronal acetate, *p*H 7.3 (*7*).

In a series of 12- by 75-mm tubes were placed 0.1 ml of this aged Na_2-EDTA plasma; 0.1 ml of Asolectin (0.005 percent); 0.05 ml of barium sulfate adsorbed bovine serum, diluted 1 to 80; and 0.05 ml of either normal or unknown plasmas in serial dilutions. The coagulation times for these mixtures after recalcification with 0.1 ml of 1/70*M* $CaCl_2$, were recorded, and the values for tubes containing the unknown plasma were compared with those for tubes containing serial dilutions of normal plasma (see Table 1). The average recalcification time for such a system, when buffer is substituted for the unknown plasma, is 14 minutes; the time is shortened to 5 minutes or less (normal, 4¼ minutes) when any one of the following is added instead: platelet-poor normal plasma; plasma from a patient receiving Dicumarol; Seitz-filtered normal plasma; barium sulfate adsorbed normal plasma; and plasmas from patients congenitally deficient in PTA (one patient), Hageman factor (one patient), PTC (three patients), proconvertin (one patient), and proaccelerin (one patient). However, in none of six patients diagnosed by the method of Soulier and Larrieu (*2*)

Table 1. Coagulation time with artificial reagent to which the various plasmas listed were added prior to recalcification.

Plasma	Av. time (min)
Normal, platelet-poor	4¼
Normal, Dicumarol	3
Normal, Seitz-filtered	3¾
Normal, $BaSO_4$-adsorbed	2½
From PTA-deficient patient	4¾
From individual with Hageman trait	3¾
From PTC-deficient patient No. 1	4¼
From PTC-deficient patient No. 2	4¼
From PTC-deficient patient No. 3	4½
From proconvertin-deficient patient	2½
From proaccelerin-deficient patient	5
From AHG-deficient patient No. 1	6¼
From AHG-deficient patient No. 2	7
From AHG-deficient patient No. 3	8
From AHG-deficient patient No. 4	8
From AHG-deficient patient No. 5	10¼
From AHG-deficient patient No. 6	15
Veronal-acetate buffer (*p*H 7.3)	14