

## ROLE OF HEPATIC ANION-BINDING PROTEIN IN BROMSULPHTHALEIN CONJUGATION\*

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The transfer of Bromsulphthalein (BSP) (Hynson, Westcott, and Dunning, Inc., Baltimore, Md.) from plasma to hepatocyte may be mediated in part by a protein that can be isolated from the cell supernatant fraction of a liver homogenate. By virtue of its binding capacity for a variety of organic anions, including BSP, this protein, designated "Y" protein (1) or ligandin (2), is believed to be a determinant of the hepatic uptake process. This view is supported by the association of "physiological" jaundice with a low concentration of Y protein in fetal and neonatal liver (3, 4), as well as phylogenetic evidence demonstrating a correlation between the rate of clearance of BSP from plasma and the presence of Y protein in liver (5).

BSP is excreted in bile mainly as the glutathione conjugate that is synthesized in the liver (6). The conjugation step is considered to be rate-limiting in the overall hepatic transport of BSP from plasma to bile (7, 8). Liver cytosol has been shown to contain a soluble enzyme that catalyses the conjugation of BSP with glutathione—the glutathione *S*-aryltransferase enzyme (9, 10).

During studies of the binding of BSP and other anions to Y protein, duplicating precisely the conditions described in detail by Levi et al. (1), BSP conjugation with glutathione was found to occur. Further evaluation of this phenomenon, which forms the basis of this report, has led us to the conclusion that Y protein may act by both binding and conjugating BSP.

### *Materials and Methods*

The liver donors were albino Wistar strain rats weighing approximately 250 g. The livers were perfused *in situ* with 0.01 M phosphate buffer, pH 7.4, 0.25 M sucrose. 25% liver homogenates were prepared in the same buffer and the supernatant fraction was harvested after centrifuging at 100,000 *g* for 60 min.

Gel filtration was performed on Sephadex G-75 (Pharmacia, Uppsala, Sweden) columns 30 × 2.9 cm using 0.01 M phosphate buffer, pH 7.4, containing 0.1 M NaCl as mobile phase. Flow rate was 28 ml/h, 10 fractions/h.

BSP was obtained from Vitarine Co., Inc., New York, and [2-<sup>3</sup>H]glycine glutathione was obtained from New England Nuclear, Boston, Mass. (specific radioactivity, 285 mCi/μmol).

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Glutathione *S*-aryltransferase activity was measured using either [ $^{35}$ S]BSP and glutathione as cosubstrates (9) or 3,4-dichloronitrobenzene and glutathione as cosubstrates (10).

#### RESULTS

Using BSP binding as a protein marker, three protein fractions may be identified with elution vol of 75, 95, and 135 ml, respectively (Fig. 1). The second protein corresponds to Y protein. [ $^3$ H]Glycine glutathione was eluted as two peaks: a minor peak (elution vol 75 ml) and a large symmetrical peak (elution vol 95 ml). The latter peak is shown to correspond closely with the presence of BSP bound to Y protein (Fig. 1). Since a similar pattern of glutathione binding was also found in the absence of BSP, the glutathione binding does not represent bound BSP-glutathione conjugate.

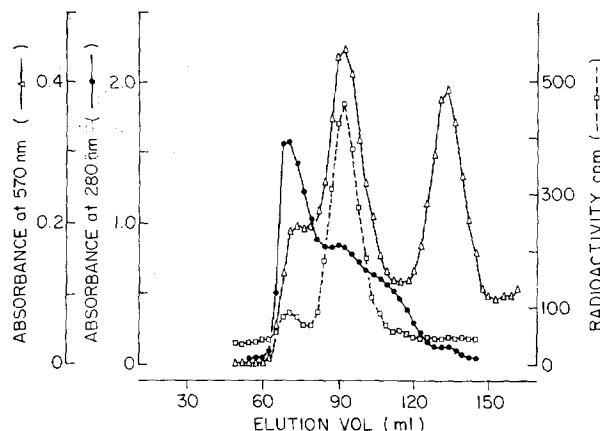


FIG. 1. Elution pattern from Sephadex G-75 column of liver cell supernatant (4.0 ml) with added BSP (4.0 mg) and [ $^3$ H]glycine glutathione (2.5  $\mu$ Ci).

Fractions corresponding to elution vol 65–80, 81–105, 106–125, and 125–150 ml were combined and glutathione *S*-aryltransferase activity was assayed using BSP as substrate (Table I). Activity greater than control was present only in the combined fractions corresponding to Y protein.

When glutathione *S*-aryltransferase activity was assayed with 3,4-dichloronitrobenzene in each individual fraction from the Sephadex column, a close correspondence with the elution pattern of [ $^3$ H]glycine glutathione was found (Fig. 2). Enzyme activity was found only in the fractions corresponding to Y protein.

#### DISCUSSION

Sephadex gel filtration permits separation of proteins according to their molecular weights (11). The addition of the organic anion, BSP, to the hepatic supernatant fraction undergoing molecular sieving has led to the identification

TABLE I  
Conjugation of BSP and Glutathione by Liver Supernatant Protein Fractions

Elution vol <i>ml</i>	Radioactivity [ <sup>35</sup> S] <i>cpm</i>		Percentage conjugation <i>%</i>
	BSP	BSP conjugate	
65-80	1,205	64	5.0
81-105	738	743	50.2
106-125	1,697	103	5.7
125-150	1,361	80	5.5

[<sup>35</sup>S]BSP (1 mM) and reduced glutathione (8 mM) were incubated in 0.01 M phosphate buffer, pH 7.4, at 37°C for 60 min together with an aliquot of the protein-containing fractions. Conjugated and unconjugated BSP were separated by thin-layer chromatography and radioactivity was estimated by liquid scintillation counting. Nonenzymic conjugation of BSP and glutathione produces approximately 5% conjugation under these conditions.

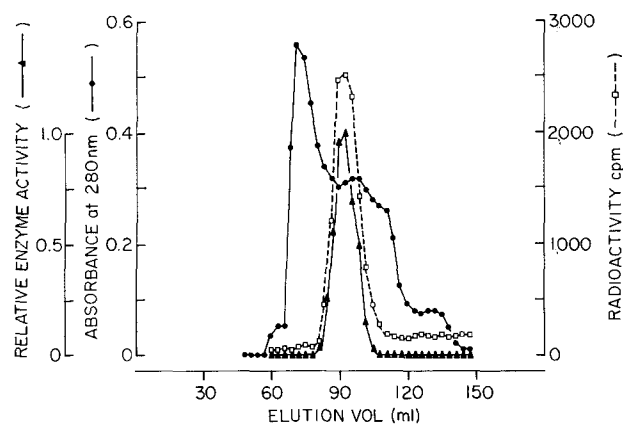


FIG. 2. Elution pattern of liver cell supernatant (2.0 ml) with added [<sup>3</sup>H]glycine glutathione (2.5  $\mu$ Ci). The maximum enzyme activity has been arbitrarily given a value of 1.0 and activity present in each fraction is plotted as a proportion of this maximum.

of a series of anion-binding proteins, one of which has been called Y protein (1) or ligandin (2). We have demonstrated that the elution volume of the BSP marking Y protein and the elution volume of glutathione are the same. This suggests that glutathione binds to the same protein as BSP and that glutathione may therefore be used as an alternative marker for Y protein during gel filtration.

Since BSP and glutathione are cosubstrates for the hepatic supernatant enzyme, glutathione *S*-aryltransferase, the finding of nearly superimposable peaks for these two substances corresponding to hepatic supernatant Y protein suggested the possibility that enzymic activity might reside in these fractions. Using two different assay systems, this enzyme activity was found to be

present in those fractions containing Y protein, and the pattern of activity corresponded with the pattern of glutathione; no activity was detected in any other fractions (Fig. 2). We are thus led to conclude that a close relationship, if not identity, exists between Y protein and glutathione *S*-aryltransferase. Further purification of the enzyme will permit us to make an exact comparison of its physical and chemical properties with those published for Y protein (2).

This newly proposed function for Y protein as a glutathione-conjugating enzyme should lead to a reappraisal of its role in the hepatocellular uptake of organic anions from plasma. The significance of binding of anions other than BSP must be assessed with particular reference to possible inhibition of enzymic activity as well as to other anions acting as substrates for the enzymes. For example, bilirubin and cholecystographic agents have been shown to be bound by Y protein and thus might inhibit the rate-limiting glutathione conjugation step in BSP transport. This would provide an explanation for the clinical impairment of plasma BSP clearance seen in liver disease or during biliary tract radiography. Thus, ligandin may participate in the hepatic transport process in two ways: as an intracellular binding protein in the hepatic uptake of organic anions and as an enzyme that further metabolizes certain of these compounds.

#### SUMMARY

Using gel filtration, the binding of both glutathione and Bromsulphthalein (BSP) to a liver-soluble protein was found to be identical. BSP-conjugating activity (glutathione *S*-aryltransferase) was present only in the fractions corresponding to the two protein-bound markers. Using a highly sensitive assay, with 3,4-dichloronitrobenzene, the pattern of glutathione *S*-aryltransferase activity was found to coincide with Y protein. This evidence suggests that Y protein, or ligandin, has a dual role in hepatic transport: a specific enzymic function in the conjugation of certain anions with glutathione in addition to a transport function in the intracellular binding of organic anions.

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