PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 234–237 (1975)

Hepatic Glutathione S-Transferases: Identification by Gel Filtration and *in Vitro* Inhibition by Organic Anions[1-5] (38779)

NEIL KAPLOWITZ, JOHN KUHLENKAMP, AND GIL CLIFTON
(Introduced by R. Schmid)

Clinical Investigation Center, Naval Regional Medical Center, Oakland, California 94627

An hepatic protein of molecular weight approximately 45,000, known as Y protein or ligandin, is believed to be a cytoplasmic acceptor in the uptake of organic anions from plasma to hepatocyte (1, 2). Binding of the organic anion, sulfobromophthalein sodium (BSP), has been used as a convenient marker to identify Y protein in the gel filtration of liver cytosol (1). BSP is converted in the liver to a glutathione conjugate, a reaction believed to be catalyzed by the soluble enzyme, glutathione S-aryltransferase (3–5). A close relationship between Y protein and glutathione S-aryltransferase activity in liver cytosol has been proposed previously on the basis of the finding of an identical elution volume in gel filtration for both BSP conjugating activity and BSP binding (6).

In addition to glutathione S-aryltransferase, other enzymic activities in liver, collectively referred to as the glutathione S-transferases (7, 8), catalyze glutathione conjugation with certain aralkyl (9), epoxide (9, 10), and alkyl (11) substrates. The experiments described in this communication were designed to extend previous work by examining the relationship of four glutathione S-transferase activities to BSP and glutathione binding in gel filtration as well as to examine by enzymic inhibition the binding of organic anions by this group of enzymes.

Materials and Methods. Liver donors were albino Sprague–Dawley rats weighing 160–180 g. The livers were perfused in situ with 0.01 *M* phosphate buffer, pH 7.4, 0.25 *M* sucrose. Twenty per cent liver homogenates were prepared in the same buffer, and the supernatant fraction (cytosol) was harvested after centrifuging at 105,000 *g* for 60 min.

Gel filtration was performed on Sephadex G-75 (Pharmacia, Uppsala, Sweden) columns 35 × 2.5 cm using 0.01 *M* phosphate buffer, pH 7.4 as mobile phase. Flow rate was 28 ml per hr, 10 fractions per hr.

BSP was obtained from J. T. Baker Chemical Co., Philipsburg, NJ, indocyanine green (ICG) from Hynson, Westcott and Dunning, Inc., Baltimore, MD. and bilirubin from Sigma Chemical Co., St. Louis, MO [2-^{3}H]-glycine–glutathione was obtained from New England Nuclear, Boston, MA (specific radioactivity, 250 mCi per μmol).

Glutathione S-aryl-, S-epoxide-, and S-aralkyltransferase activities were measured kinetically (5, 8, 10) in fractions collected from Sephadex G75 and in 105,000 *g* supernatant using reduced glutathione (Sigma Chemical Co.) and the following substrates, respectively: 3,4-dichloronitrobenzene (Aldrich Chemical Co., St. Louis, MO), 1,2-epoxy-3-(*p*-nitrophenoxy) propane (Eastman Kodak Co., Rochester, NY), and *p*-nitrobenzyl chloride (Aldrich Chemical Co.). Glutathione S-alkyl-transferase activity was measured according to the method of Johnson (11) using reduced glutathione and [^{14}C]methyl iodide (New England Nuclear, specific radioactivity 4.86 μCi per μmole) as cosubstrates.

The "least squares" regression line and

[1] Presented in part at ASCI/AFCR Meeting, Atlantic City, New Jersey, May 5, 1974.

[2] This study was supported through funds provided by the Bureau of Medicine and Surgery, Navy Department, for CIP 4-48-366.

[3] The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large.

[4] Proofs should be sent to: LCDR Neil Kaplowitz, MC, USNR, Clinical Investigation Center, Naval Regional Medical Center, Oakland, CA. 94627.

[5] Address reprint requests to: Publications Office, Clinical Investigation Center, Naval Regional Medical Center, Oakland, CA. 94627.

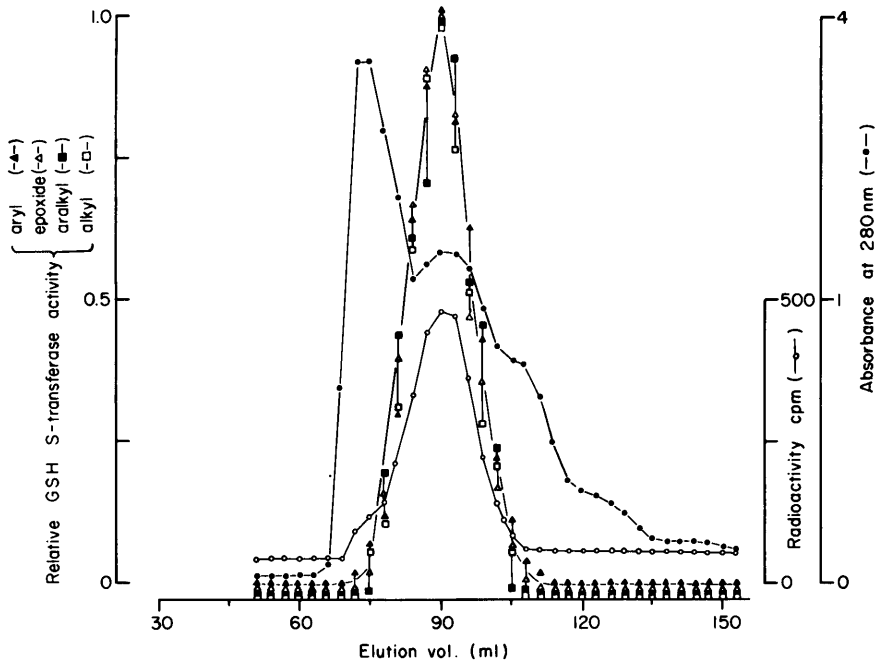

FIG. 1. Gel filtration of liver cytosol (4.0 ml) with [^{3}H]glycine glutathione (2.0 μCi). The maximum enzymatic activity for each glutathione S-transferase activity has been arbitrarily given a value of 1.0, and activity present in each fraction is plotted as a proportion of this maximum. A superimposable elution pattern is seen for the four GSH transferase activities and [^{3}H]glycine glutathione.

correlation coefficient (12) were calculated for each set of enzyme inhibition data.

Results. When individual fractions collected from a single gel filtration run of liver cytosol were assayed for four glutathione S-transferase activities, an identical elution volume (90 ml) was obtained for each of the enzymic activities (i.e., glutathione S-aryl-, S-epoxide-, S-alkyl-, and S-aralkyltransferase (Fig. 1). Repeated gel filtrations under identical conditions using cytosol from different animals consistently showed the same elution volume for the enzymic activities. Bound [^{3}H]glycine glutathione was eluted in a pattern superimposable with the four glutathione S-transferases (Fig. 1). In addition, the same elution volume (90 ml) was found for BSP binding under the same column conditions.

Organic anion binding by the glutathione S-transferases was investigated using enzyme kinetics with BSP, ICG, and bilirubin as inhibitors of the activities in liver cytosol. These three organic anions were found to be competitive inhibitors of the four activities. An example of the data obtained is represented in Fig. 2 in the form of a Dixon plot (13) showing bilirubin as a competitive inhibitor of glutathione S-aryltransferase activity. The inhibitor constants (K_i) listed in Table I have a wide range of values (0.0308–152.0 $\times$ 10^{-6} M). The inhibitor constant in each instance was calculated using the regression equations from the Dixon plot and represents the negative x–coordinate value corresponding to the intercept of the lines. ICG was the most potent inhibitor of the S-aryl- and S-aralkyltransferase activities (K_i 0.479 and 0.031 $\times$ 10^{-6} M, respectively). BSP was the greatest inhibitor of the S-epoxide and S-alkyltransferase activities (K_i 13.1 and 1.60 $\times$ 10^{-6} M, respectively). Bilirubin showed the least inhibition of the three organic anions for the four enzymic activities.

Discussion. Previous gel filtration experiments (6) have demonstrated similarities in molecular weight and BSP binding for glutathione S-aryltransferase, the cytoplasmic enzymic activity responsible for BSP

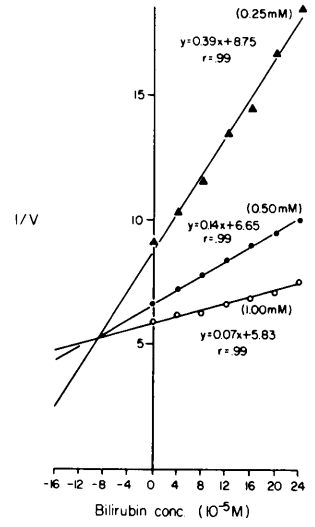

FIG. 2. Competitive Dixon plot of the inhibition of glutathione S-aryltransferase by bilirubin. Reaction mixtures contained 3,4-dichloronitrobenzene (0.25, 0.50, or 1.00 m*M*), reduced glutathione (10 m*M*), and bilirubin (0–0.24 m*M*) in pH 8.0, 0.1 M phosphate buffer (2.05 ml) at 37°. The reaction was initiated by the addition of 10 μl liver cytosol.

TABLE I. COMPETITIVE INHIBITION OF THE GLUTATHIONE S-TRANSFERASES BY ORGANIC ANIONS.

Enzyme activity	Inhibitor constants (10^{-6} *M*)		
	BSP	ICG	Bilirubin
S-Aryl-	44.6	0.479	95.2
S-Epoxide	13.1	14.1	31.5
S-Aralkyl-	3.34	0.031	12.9
S-Alkyl-transferase	1.60	50.7	152.0

conjugation with glutathione, and Y protein, the cytoplasmic BSP binding protein. In these experiments a single major peak of glutathione binding in liver cytosol was observed, corresponding exactly to the elution volume of both Y protein and glutathione S-aryltransferase activity. Therefore, this single glutathione peak has been useful as an alternate marker for the location of gel filtration fractions containing BSP binding activity. However, although only one major glutathione peak has been observed, glutathione is utilized as a substrate for a group of hepatic cytosolic S-transferase activities: glutathione S-alkyl-, S-aralkyl-, S-epoxide-, and S-aryltransferases (4, 7–11). Studies were therefore performed in an attempt to explain the relationship between BSP binding to Y protein, glutathione binding to cytosolic protein, and the glutathione S-transferase enzyme system.

The four major glutathione S-transferase activities were eluted in gel filtration in a pattern superimposable with both BSP binding and glutathione binding (Fig. 1). BSP binding has been used as a marker to identify Y protein in gel filtration (1). Our findings indicate a close correspondence in molecular weight or size between the enzymes responsible for the four glutathione S-transferase activities and Y protein. Moreover, the enzyme inhibition studies demonstrate that these soluble hepatic enzymes are similar to Y protein or ligandin in the property of binding organic anions. Three organic anions, BSP, ICG, and bilirubin, which undergo competitive hepatic transport (14–16) and also bind to ligandin (1, 2), were tested. Each was found to inhibit competitively the four glutathione S-transferase substrate activities studied, presumably by interacting with the active site responsible for each activity. Of these three organic anions, only BSP is a substrate for glutathione conjugation (3–5) and only for the glutathione S-aryltransferase (5); ICG is excreted unchanged into bile (16), and bilirubin is metabolized principally to a diglucuronide (17). Thus, these inhibition studies demonstrate that the glutathione S-transferases, like ligandin, are capable of binding organic anions which are not necessarily metabolized by these enzymes.

Pabst *et al.* (8) have prepared several homogeneous GSH-transferases which were shown to have heterogeneous enzymic activities. However, although each homogeneous protein catalyzed reactions with several different substrates, 3,4-dichloronitrobenzene transferase activity was found to be almost completely separable from epoxide and methyl iodide transferase activities (8). Thus, the four transferase substrates which we have employed represent the activities of at least two distinct proteins or groups of enzymes which have been identified by Pabst *et al.* (8). Therefore, one can conclude that two or more glutathione S-transferase proteins have organic anion binding properties. Ligandin cannot be the

only organic anion-binding protein whether or not it is ultimately shown to be a member of this family of enzymes.

Enzyme kinetic studies employing whole cytosol have been useful not only in qualitatively identifying the interaction of organic anions with these enzymes as shown in this report, but also in comparing the inhibitory affinities of various organic anions with respect to a given substrate activity. However in order to determine the relative importance of individual enzymes and/or ligandin in organic anion binding, binding studies employing quantitatively recovered homogeneous proteins are needed. In this report using a crude mixture of enzymes, ICG was found to be the most potent inhibitor of the 3,4-dichloronitrobenzene and *p*-nitrobenzyl chloride transferase activities, whereas, BSP was the most potent of the three organic anions studied as inhibitor of the methyl iodide and epoxide transferase activities. These experiments suggest that the enzyme(s) most inhibited by ICG are different from those most affected by BSP. Therefore, specific organic anions may preferentially bind to one or more of the enzymes so that the protein responsible for the cytoplasmic binding of ICG may be different from that responsible for BSP binding.

On the basis of both the identification of the glutathione S-transferases in the same gel filtration fraction having BSP binding activity marking the presence of Y protein and the qualitative demonstration of the capacity of these enzymes to bind inhibitory organic anions, it is tempting to speculate that the organic anion-binding protein, ligandin, is one of this group of enzymes. Thus, our previous suggestion concerning the striking similarities between glutathione S-aryltransferase and ligandin (6) must now be expanded to include the group of glutathione S-transferases.

From a molecular biological view it is intriguing to consider the possibility that this enzyme system may have a unique dual purpose. The transferases are known to have specific enzymic functions in catalyzing glutathione conjugation with certain compounds including some organic anions. In addition, these enzymes may play a role in hepatic transport as a group of cytoplasmic acceptors of organic anions.

Summary. In the gel filtration of 100,000 *g* rat liver supernatant, four major glutathione S-transferase activities, S-aryl-, S-epoxide-, S-aralkyl-, and S-alkyltransferase, were identified as having an elution volume identical to that of fractions exhibiting either glutathione or sulfobromophthalein sodium binding. The organic anions, sulfobromophthalein sodium, indocyanine green, and bilirubin, were found to be competitive inhibitors of the four glutathione S-transferase activities. These findings indicate that the glutathione S-transferases bind organic anions and, as a group, have a similar molecular weight to a known organic anion-binding protein. It is proposed that these enzymes also serve nonenzymically as a group of binding proteins in the hepatic cytoplasmic transport of organic anions.

1. Levi, A. J., Gatmaitan, Z., and Arias, I. M., J. Clin. Invest. **48,** 2156 (1969).
2. Litwack, G., Ketterer, B., and Arias, I. M., Nature (London) **234,** 466 (1971).
3. Combes, B., and Stakelum, G. S., J. Clin. Invest. **40,** 981 (1961).
4. Javitt, N. B., Wheeler, H. O., Baker, K. J., et al., J. Clin. Invest. **39,** 1570 (1960).
5. Booth, J., Boyland, E., and Sims, P., Biochem. J. **79,** 516 (1961).
6. Kaplowitz, N., Percy-Robb, I. W., and Javitt, N. B., J. Exp. Med. **138,** 483 (1973).
7. Boyland, E., and Chasseaud, L. F., Adv. Enzymol. **32,** 173 (1969).
8. Pabst, M. J., Habig, W. H., and Jakoby, W. B., Biochem. Biophys. Res. Commun. **52,** 1123 (1973).
9. Boyland, E., and Williams, K., Biochem. J. **94,** 190 (1965).
10. Fjellstedt, T. A., Allen, R. H., Duncan, B. K., et al., J. Biol. Chem. **248,** 3702 (1973).
11. Johnson, M. K., Biochem. J. **98,** 44 (1966).
12. Batson, H. C., "An Introduction to Statistics in the Medical Sciences," p. 54. Chicago Medical Book Co., Chicago (1956).
13. Dixon, M., Biochem. J. **55,** 170 (1953).
14. Hunton, D. B., Bollman, J. L., and Hoffman, H. N. II, J. Clin. Invest. **40,** 1648 (1961).
15. Hargreaves, T., and Lathe, G. H., Nature (London) **200,** 1172 (1963).
16. Wheeler, H. O., Cranston, W. I., and Meltzer, J. I., Proc. Soc. Exp. Biol. Med. **99,** 11 (1958).
17. Fevery, J., Van Damme, B., Michiels, R., et al., J. Clin. Invest. **51,** 2482 (1972).

Received September 6, 1974. P.S.E.B.M. 1975, Vol. 149.