significant that an antibody which was undetectable in maternal rat blood following intravenous administration, has proven to be so potent a teratogenic agent. From both theoretical and practical standpoints, it would be most important to understand the qualitative and quantitative mechanisms by which this biologically prepared and altered protein induces malformations.

Summary. Rabbit anti-rat kidney sera, in a prescribed dosage, injected intravenously into pregnant rats on the 8th day of gestation, resulted in severe congenital malformations in 100% of the fetuses. Larger doses caused complete fetal resorption. These antisera may produce teratogenesis by interfering with placental function.

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Biosynthesis of a Sulfobromophthalein Mercaptide with Glutathione.* (26391)

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Although glutathione mercaptides have long been suggested as intermediates in mercapturic acid synthesis(1-4), isolation and identification of a glutathione mercaptide from biological fluids has proved difficult.

The dye sulfobromophthalein (BSP) is rapidly taken up by the liver and secreted into the bile as pigmented metabolites(5-8). The main metabolite appearing in human bile has been identified as an unacetylated mercaptide formed through the –SH group of cysteine(9). The consistent demonstration of both glycine and glutamic acid in acid hydrolysates of this metabolite(5,7-9) has suggested that it may be a BSP-glutathione mercaptide(9,10). Positive identification of the glutathione mercaptide was difficult since both glycine and glutamic acid were found in acid hydrolysates of identically prepared BSP-free fractions from normal bile(5,9).

The present report describes the successful incorporation of isotopic glycine, glutamic acid and cysteine into the major BSP metabolite synthesized by the isolated perfused liver of the rat. The results indicate that BSP is excreted into bile primarily as a mercaptide with glutathione.

Methods. Intact livers were taken from Long-Evans male rats, weighing 400-450 g, and perfused(11). At 1 hr 50-125 μc of either glycine-U-C^{14}, glutamic acid-U-C^{14}, glutamine-U-C^{14}, cystine-S^{35} or glutathione-S^{35} (Schwartz Laboratories) was added to the perfusate. After 30 min equilibration, bile to be used in the controls was collected for 30 min and immediately frozen. BSP, 20 mg, was then introduced into the perfusate and equilibrated for 30 min. Experimental bile was collected during the subsequent 30 min interval. BSP levels were determined by the technic of Gaebler(12). Samples of bile or bile fractions, 10-50 μl, were plated and counted with an end-window Geiger-

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Müller counter. Duplicate samples varied less than 8% and corrections for self-absorption proved to be minimal.

Fractions of equal radioactivity from both experimental and control bile were purified identically. Samples were initially extracted with 3 volumes of acetone, and the extracts were column chromatographed as described previously(9). The first fraction obtained by employing acetone/water/concentrated NH_4OH (12.5:11.5:1 v/v) as solvent contained the bile pigments, free BSP not metabolized by the liver, some of the minor, less polar metabolites found in rat bile, and free amino acids and peptides. About 88-96% of labeled cystine, glutathione, glutamic acid, glutamine and glycine could be recovered in this fraction. The BSP metabolites were then eluted from the column, using acetone/water (1:9 v/v) as solvent. This second fraction was concentrated by rotary evaporation under reduced pressure at 40°, dried *in vacuo,* redissolved in a minimal quantity of water and chromatographed on washed Whatman 3 MM paper(9) in an ascending system employing n-butanol/water (1.48:1 v/v) made into a single phase solution with 0.27 volume of acetic acid. The metabolite fraction located by exposure to ammonia fumes was eluted with water and rechromatographed in *tert* butanol/water (1.73:1 v/v). Serial segments of each chromatogram from origin to solvent front were cut out and counted. The incorporation of label into the metabolized fraction was compared to that in the remaining portion of the chromatogram.

Both the purified metabolites and comparable control fractions were hydrolyzed in 6 N HCl at 120° for 15 hr. HCl was removed by repeated evaporation to dryness of water solutions. The labeled products were separated and their mobilities compared to those of reference amino acids on Whatman No. 1 paper employing the *tert* butanol system.

In 4 nonisotopic experiments the metabolite was isolated from the bile and serially purified as described above. Total BSP and free amino nitrogen content(13) were determined on aliquots taken before and after acid hydrolysis. The hydrolysate was chromatographed in the *tert* butanol/water system. The fractions corresponding in mobility to glycine, glutamic acid and cysteine were eluted with water and their molar relationship was determined with ninhydrin.

Results. Acetone extracts of experimental bile contained 3 chromatographically distinct forms of the secreted BSP(5). Both free BSP and a fast-moving metabolic product were removed in the initial eluate on the alumina column. Thus, the slow-moving product, which in the isolated perfused liver system represented 85-90% of the secreted BSP, was the only fraction investigated.

When either cystine-S^{35}, glutathione-S^{35}, glutamic acid-C^{14}, glutamine-C^{14}, or glycine-C^{14} was perfused through the isolated liver preparation (Table I), approximately 25-50% of the label appearing in the experimental bile was associated with the BSP metabolite fraction from the alumina column. Subsequent purification of the metabolite by 2 paper-chromatographic systems removed only small amounts of the individual label. Fractions of control bile after similar purification showed no significant isotopic activity.

The hydrolyzed BSP metabolite obtained after glycine perfusion yielded a single major radioactive spot representing 90% of the total label and corresponding in mobility to reference glycine. Similarly, hydrolysis of glutamic acid-C^{14} and glutamine-C^{14} metabolites resulted in the appearance of radioactivity in the glutamic acid position in the chromatogram. Hydrolysis and chromatography of S^{35}-containing metabolites from liver perfused with cystine or glutathione produced ill-defined S^{35}-active fractions at the origin, at the Rf of cysteine (0.15) and the degraded pigmented moiety (0.92), and at the solvent front.

The BSP and free amino nitrogen content of the purified unlabeled metabolite before and after acid hydrolysis is shown in Table II. Control bile fractions, although ninhydrin-negative before hydrolysis, contributed some ninhydrin-positive material after treatment with HCl (probably arising from impurities in the chromatographic paper). After

TABLE I. Levels of Isotopic Amino Acids and Glutathione in BSP Metabolites and Control Fractions at Successive Stages of Purification. Samples of control and experimental bile containing equal radioactivity (taken as 100%) were serially purified by column and paper chromatography. Results are reported as percentage of radioactivity in the metabolite fractions.

	Acetone/water eluate from alumina column		Paper chromatography			
			n-butanol water/HAc*		*tert* butanol/water†	
	Control	Exp.	Control	Exp.	Control	Exp.
Cystine-S^{35}	10	29	1	21	0	19
	10	27	1	23	0	22
	10	26	1	20	0	18
Glutathione-S^{35}	10	24	1	22	0	20
	9	28	0	24	0	23
Glutamic acid-C^{14}	5	52	0	49	0	47
	8	34	2	28	0	28
Glutamine-C^{14}	5	38	0	33	0	30
Glycine-C^{14}	7	60	1	21	0	17
	6	40	0	26	0	25

* Metabolite fraction: Rf = 4.0-5.0.

† Metabolite fraction: Rf = 4.0-4.5.

correction for these impurities, approximately 3 μM of amino nitrogen were found for each μM of the BSP present before hydrolysis.†

The mean molar ratio of glycine, glutamic acid and cystine, determined after chromatographic separation of the hydrolysates, was 1:0.91:0.18 (Table III). In agreement with other reports(5,7,8) no other amino acids were detected, except alanine in small amounts.

Effect of cysteine concentration on synthesis of glutathione mercaptide. To determine whether cysteine may compete with glutathione for the synthesis of BSP mercaptides, 250 mg of cysteine hydrochloride was dissolved in minimal water. After being adjusted to pH 7.4, the solution was added to the perfusate of a glycine-C^{14} experiment. (Higher concentrations of cysteine proved toxic to the perfused liver.) Neither the percentage of secreted BSP appearing as the major slow-moving fraction (91%) nor the uptake of glycine-C^{14} into the purified metabolite (23%) was affected by the added cysteine.

TABLE II. Appearance of Amino Nitrogen after Acid Hydrolysis of Purified BSP Metabolite. Unlabeled BSP metabolite purified by the 3 chromatographic procedures described in text was hydrolyzed in 6 N HCl for 20 hr at 120°. After removal of HCl from the hydrolysate, total amino nitrogen was determined by method of Moore and Stein(13).

Perfusion	Purified control fraction μM N	Purified metabolite of BSP μM N	Metabolite minus control μM N	BSP in metabolite sample μM	N/BSP, μM/μM
1	1.5	2.6	1.1	.4	2.8
2	1.1	2.4	1.3	.4	3.3
3	1.1	5.5	4.4	1.3	3.4
4	1.2	2.2	1.0	.3	3.3

† This calculation assumes that free and conjugated BSP have the same molecular extinction coefficient. Accurate determination of the constant for the conjugate must await its availability in crystalline, pure form.

Discussion. Incorporation of glycine-C^{14}, glutamic acid-C^{14} and cysteine-S^{35} into the major BSP metabolite appearing in bile from isolated perfused rat liver and their absence from comparable control fractions strongly support the previous suggestions that the pigment is conjugated, at least in part, by the liver with glutathione(9).

Since both C^{14}-glycine and C^{14}-glutamic acid were isolated intact after hydrolytic degradation of the respective metabolites resulting from perfusion of the two amino acids, the isotopes present in the metabolite could not have resulted from degradation of the C^{14}-labeled amino acids and the ultimate entrance of their C^{14} fragments into the carbon pool and thence into the carbon skeleton of cysteine itself(14).

That the mercaptide conjugate of BSP

TABLE III. Recovery of Glycine, Glutamic Acid and Cysteine after Acid Hydrolysis of BSP Metabolite. Hydrolyzed metabolite (see Table II for details), after removal of HCl, was subjected to paper chromatography in the *tert* butanol/water system. Fractions corresponding in mobility to glycine, glutamic acid and cysteine were eluted with water and their amino nitrogen was determined. Results are given as μg N in sample chromatographed.

	Glycine		Glutamic acid		Cysteine		Ratio after subtracting control values
Perfusion	Control	Exp.	Control	Exp.	Control	Exp.	(gly : glu : cyst)
1	4.7	14.9	5.3	13.6	0	2.5	1 : .81 : .25
2	4.1	12.0	4.6	11.8	1.8	1.8	1 : .91 : .0
3	3.4	30.8	3.5	29.4	0	4.8	1 : .94 : .17
4	5.3	12.0	5.2	11.8	0	2.0	1 : .98 : .30

contains glutathione is further suggested by the observations that a) 3 M of ninhydrin-positive amino groups per M of BSP were liberated after acid hydrolysis, b) except for small amounts of alanine (presumably arising as a hydrolytic product of cysteine), only glycine, glutamic acid and cysteine were detectable, and c) glycine and glutamic acid were present in equal molar relation. (Since cysteine is extensively degraded during hydrolysis with mineral acids(9), the low recovery of this amino acid was not surprising.) Incorporation of glutamine into the glutamic acid moiety of the conjugate was expected since glutamine is rapidly transferred into liver cells where it can be hydrolyzed to glutamic acid by glutaminase(15). Glutathione-S^{35} also acted as a precursor for glutathione conjugation, although from these experiments alone it was not possible to conclude whether the glutathione was incorporated intact into the glutathione mercaptide or whether it acted solely as a source of cysteine which was then incorporated into some activated glutathione precursor.

During this investigation, Booth, Boyland and Sims(16) reported successful synthesis of a naphthalene glutathione conjugate in rat liver slices. The results of their work and our isotopic studies leave little doubt that the liver synthesizes mercaptide conjugates containing glutathione. The following scheme(17) for mercapturic acid synthesis has been suggested:

R + glutathione → R-glutathione → R-cysteine + glycine + glutamic acid → R-acetylcysteine (mercapturic acid).

Studies *in vivo* have demonstrated that enzymes are available for hydrolysis of a glutathione mercaptide to a cysteine mercaptide(4,17). The primary site of this enzymatic activity may be the kidney(16). Although small amounts of BSP metabolites have been found in urine from hepatectomized dogs(18), BSP is normally excreted directly from the liver into the bile, which may explain why it is found primarily as a glutathione conjugate.

The observation that high levels of cysteine added to the perfusate did not significantly affect the synthesis of BSP glutathione supports the concept that cysteine and glutathione do not compete directly for molecules to be conjugated but instead that mercaptide conjugation with glutathione is a specific, enzymatically controlled step preceding mercapturic acid synthesis.

Summary. The incorporation of glycine-U-C^{14}, glutamic acid-U-C^{14}, glutamine-U-C^{14}, cystine-S^{35} and glutathione-S^{35} into the major mercaptide conjugate of sulfobromophthalein (BSP) was studied in bile from isolated perfused rat liver. Incorporation of label from each of the amino acids was demonstrable in the purified metabolite obtained after 3 serial chromatographic steps, whereas control fractions were negative. Acid hydrolysis of the metabolite and subsequent paper chromatography revealed that the amino acids had been incorporated intact, that 3 M of amino nitrogen were released for each M of BSP, and that glycine and glutamic acid were in equimolar ratio (cysteine was degraded during hydrolysis and could not be quantitatively recovered). High concentrations of cysteine in the perfusate did not compete with glutathione for the synthesis of BSP mercaptide. It was concluded that BSP

is secreted into bile, at least in part, as a mercaptide conjugate with glutathione.

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Urea Derivatives as Fibrinolysis Promoting Agents.* (26392)

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During the search for synthetic compounds to enhance the induction of fibrinolysis in human plasma by urokinase, Cibalgin,® an analgesic drug, was found to be active. Subsequent testing showed that enhancement was derived from 2 components, urethan and ethylurea. A variety of other urea derivatives was then found with similar properties. Controls revealed that some of these compounds slowly dissolved plasma clots in absence of urokinase. We therefore undertook to determine whether small amounts of such non-toxic chemicals could induce activation of the fibrinolytic enzyme system *in vitro*.

Material and methods. The preformed rotating standard clots were prepared from either fibrinogen or human plasma as previously described(1), except that a rotating device was used instead of a rocking device. This arrangement permits study of fibrinolysis promoting agents acting upon one well defined invariable surface of a preformed standardized clot. The disintegration products are continuously removed from the clot surface, and concentration of the agents being tested, in contrast to fibrin plate method, does not vary to a great extent. The progress of the clot lysis is read at intervals in terms of ml of digested clot.

Human plasma clots. Human ACD bank plasma (3 to 5 weeks old) or fresh plasma (1 part Na-citrate USP 0.129 M to 4 parts blood, centrifuged 5 minutes at 1500 g) was clotted by addition of 0.05 ml $CaCl_2$ (0.5 M) per ml for standard clots or by 0.01 ml thrombin for test tube studies. There was no consistent difference in the results of experiments using fresh or stored plasma.

Bovine fibrin standard clots. Armour bovine fibrinogen (fraction I from bovine plasma) was dissolved to 0.4% in borate buffer pH 7.4 and clotted by addition of 0.01 ml thrombin per ml fibrinogen solution.

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