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METABOLISM AND BILIARY EXCRETION OF SULFOBROMOPHTHALEIN IN VITAMIN A DEFICIENCY

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Abstract—Vitamin A deficiency in rats significantly increased hepatic glutathione *S*-aryltransferase activities measured *in vitro* against 1,2-dichloro-4-nitrobenzene (DCNB) and sulfobromophthalein (BSP). These changes were accompanied by a decrease in hepatic glutathione reductase activity in the deficient animals, but there were no alterations in either oxidized glutathione (GSSG) levels or the ratio of reduced glutathione (GSH) to GSSG in the liver. The relation between plasma BSP clearance, biliary BSP excretion, and the enzymatic conjugation rate of the dye were examined. Plasma clearance, hepatic uptake and hepatic storage of BSP were reduced in vitamin A deficient animals. Biliary concentration, excretion rate and cumulative excretion of BSP, on the other hand, were increased in these animals. The increases in these variables were due predominantly to increased formation of conjugated BSP. It is concluded, therefore, that the rate of the enzymatic conjugation of BSP with GSH is a rate-limiting step in the biliary excretion of the dye.

The rate of removal of sulfobromophthalein (BSP) from plasma is dependent upon a number of hepatic processes: transfer of BSP from the plasma to the liver, storage within the hepatocytes, conjugation of BSP with reduced glutathione (GSH), transfer of conjugated BSP from the liver cell into bile, and, finally, bile flow [1]. There is considerable controversy over the quantitative significance of conjugation as a factor in the rate of biliary excretion of BSP. Combes [2] reported that impairment of GSH conjugation by feeding rats a protein-free diet resulted in a reduction in the maximal rate of BSP excretion. Whelan *et al.* [3] demonstrated that dye excretion in the bile was twice as great when conjugated (BSP–GSH), rather than free, BSP was administered intravenously to rats and guinea pigs. Recently, Varga *et al.* [4] corroborated the initial observation of Combes [2], that BSP–GSH was preferentially excreted into the bile, and estimated the conjugate to have ten to thirteen times greater affinity than BSP for the biliary transport system. In rats, the decrease in total biliary BSP concentration caused by benziodarone was shown to result from a reduced excretion of BSP–GSH [5].

Although these reports suggest an important role for conjugation in BSP clearance, studies with phenobarbital have been conflicting. Klaassen and Plaa [6] reported that, although phenobarbital pretreatment increased the enzymatic conjugation of BSP and GSH, the increased conjugation did not contribute to increased biliary excretion of BSP. In their study, however, any increase in the rate of biliary excretion of the dye that resulted from the small, phenobarbital-induced increase (23 per cent) in the rate of conjugation may have been overshadowed by the much greater excretion caused by an increase in bile flow evoked by phenobarbital. In the present investigation we used vitamin A deficient rats to study (1) the effect of deficiency on the enzymatic conjugation of BSP with GSH, and (2) the role of conjugation in the plasma clearance and biliary excretion of BSP.

MATERIALS AND METHODS

Animals. Male weanling Sprague–Dawley rats (23 days old; 50–60 g) were obtained from Taconic Farms, Germantown, NY, and maintained for 6 weeks on either a vitamin A deficient diet (ICN Pharmaceuticals, Inc., Cleveland, OH, catalog No. 104646) or an identical diet supplemented with 20,000 I.U./kg of retinyl acetate.

Preparation of cytosol. The rats were killed and the livers were quickly excised and rinsed in cold KCl–Tris buffer (150 mM KCl–50 mM Tris–HCl), pH 7.4. Subsequent procedures for the preparation of cytosol were conducted at 0–4°. The livers were minced with scissors and homogenized in 2 vol. of KCl–Tris using a motor-driven Teflon and glass tissue grinder (size C, A. H. Thomas & Co., Philadelphia, PA). The resulting homogenates were diluted to 250 mg liver/ml with KCl–Tris and centrifuged at 105,000 *g* for 60 min in a Beckman L3-50 ultracentrifuge. The cytosol (supernatant fraction) was carefully removed, avoiding the floating lipid pellicle, and diluted to the desired protein concentration as described previously [7].

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Analytical procedures. Enzyme assays were conducted aerobically at 37° with conditions for zero-order kinetics regarding cofactor and substrate concentrations; activities were linear with respect to enzyme concentration and time. The GSH *S*-aryltransferase activities were determined using 1,2-dichloro-4-nitrobenzene (DCNB) [8] and BSP [9]. The glutathione reductase activity was measured according to the method of Mize and Langdon [10]. Hepatic GSH and total glutathione (GSH + GSSG)* were determined in the undiluted cytosol. Protein in the sample was precipitated by addition of an equal volume of 4% (w/v) sulfosalicylic acid and, after centrifugation at 2000 *g* for 15 min, GSH in the supernatant fluid was determined by its reaction with 5-5′-dithiobis-(2-nitrobenzoic acid) as described by Ellman [11]. The same supernatant fraction was diluted 5-fold with 0.1 M sodium phosphate buffer, pH 8.0, and a 0.05-ml aliquot was used to determine total glutathione according to the method of Tietze [12]. Hepatic vitamin A levels were measured as described by Dugan *et al.* [13]. Protein was determined by the method of Lowry *et al.* [14].

Plasma clearance and biliary excretion of BSP. Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and the common bile duct and carotid artery were cannulated with PE-10 and PE-50 tubing, respectively. The rectal temperature was maintained at 37° with heat lamps. BSP (100 mg/kg) was administered i.v. (femoral vein) as an aqueous solution (2.0 ml/kg). Blood samples (0.3 ml) from the carotid artery were collected in heparinized micro-tubes at 3, 6, 10, 15, 20, 25 and 30 min. After each sample was removed, blood was replaced with an equal volume of saline (containing 100 units/ml of heparin) infused through the arterial cannula. Bile samples were collected in pre-weighed tubes at 10-min intervals for 120 min, and the volumes estimated gravimetrically; the density of the bile was assumed to be 1.0.

Transport maximum and hepatic storage of BSP. These parameters were determined as described by Klaassen and Plaa [15]. Rats were anesthetized, and their common bile ducts and carotid arteries were cannulated as described above. In addition, a femoral vein was cannulated with PE-10 tubing. BSP was infused ($3\ mg \cdot min^{-1} \cdot kg^{-1}$) via the femoral vein as an aqueous solution (1.67 ml/hr). Blood samples (0.3 ml) were taken as before at 5, 10, 15 and 20 min after the start of the infusion. Bile samples were collected at 10-min intervals for 60 min. Plasma volumes in a separate group of control (29.7 ± 1.6 ml/kg, mean ± S.E.) and deficient (30.1 ± 0.9 ml/kg) animals (N = 5) were determined with Evan's blue dye [16].

Hepatic uptake of BSP. This was determined according to the method of Combes [2]. Rats were anesthetized and their carotid arteries were cannulated (see above). In addition, the bile ducts were ligated to prevent BSP excretion into the bile. BSP (100 mg/kg, 2 ml/kg) was injected via the femoral vein 60 min after surgery. At 15 or 45 min after injection, blood was collected in heparinized tubes from the carotid artery, and the liver removed and frozen immediately between two blocks of dry ice for subsequent analyses.

Analysis of BSP. For the estimation of BSP in blood, 0.1 ml of plasma was diluted with 6.0 ml of 0.1 N NaOH, and the absorbance determined at 576 and 414 nm. The equation (1.02 × absorbance at 576 nm) − (0.29 × absorbance at 414 nm) was used to correct for hemolysis, as reported by Iga *et al.* [17]. BSP in the bile was estimated by diluting a 0.01-ml sample with 12.0 ml of 0.1 N NaOH and measuring the absorbance at 576 nm. The hepatic content of free and conjugated BSP was determined as described by Schulze and Czok [18]. Free (R_f 0.30–0.40) and conjugated (R_f 0.00) BSP were separated on aluminum-backed silica gel 60 F_{254} thin-layer chromatography (t.l.c.) plates (0.2 mm thickness; E. M. Laboratories, Elmsford, NY) using the solvent system acetone–water–aqueous NH_3 (sp. gr. 0.88) (85:10:5, by vol.) [19]. The bands were scraped from the plates into screw-capped glass scintillation vials, and the two fractions were eluted from the silica gel by gentle shaking for 60 min with 2 ml of 75% methanol. The methanol extract was centrifuged, and the dye was estimated by diluting 1 ml of the supernatant fraction with 1 ml of 0.1 N NaOH and determining the absorbance at 576 nm. The ratio of the absorbances provided an estimate of the conjugated BSP.

Chemicals. Retinol, BSP and NADPH were purchased from the Sigma Chemical Co., St. Louis, MO. Reduced and oxidized glutathione (Grade A) and a crystalline suspension of yeast glutathione reductase (248 I.U./mg protein) were purchased from CalBiochem, San Diego, CA. 5,5′-Dithiobis-(2-nitrobenzoic acid) and 1,2-dichloro-4-nitrobenzene were obtained from the Aldrich Chemical Co., Milwaukee, WI.

RESULTS

Body and liver weights. Vitamin A deficiency results in anorexia and diminution in the rate of weight gain in young rats. Accordingly, the animals were allowed access to the respective diets *ad lib.* only during the first 4 weeks of the experiment, during which time the mean daily weight gain was similar in control and vitamin A deficient animals (Table 1). During weeks 5 and 6, however, the amount of diet supplied to the control animals was reduced in proportion to that consumed by the

Table 1. Status of experimental animals after receiving a vitamin A deficient diet*

	Control	Deficient
Mean daily growth rate (g)		
0–4 weeks	6.0 ± 0.2	5.7 ± 0.2
5–6 weeks	3.1 ± 0.2	2.9 ± 0.3
Body weights (g)	266 ± 6	255 ± 10
Liver weights (g)	11.0 ± 0.4	10.9 ± 0.3
Liver vitamin A (μg/g)	336 ± 22	< 0.5

* Animals were maintained for 6 weeks on either a vitamin A deficient diet or an identical diet supplemented with 20,000 I.U./kg of retinyl acetate (control diet). Each result is the mean ± S.E. of five to fifteen rats.

* GSSG, oxidized glutathione.

Table 2. Effect of vitamin A deficiency on hepatic glutathione levels, glutathione *S*-aryltransferease and glutathione reductase activities*

	Control	Deficient
Cytosol protein (mg/g liver)	65.6 ± 1.0	61.4 ± 2.2
Reduced glutathione (μmoles/g liver)	5.1 ± 0.6	5.3 ± 0.3
Total glutathione (μmoles/g liver)	5.6 ± 0.8	5.7 ± 0.3
Glutathione *S*-aryltransferase [nmoles·min^{-1}·(mg protein)$^{-1}$]		
1,2-dichloro-4-nitrobenzene	98.8 ± 9.6	139.5 ± 5.6†
Sulfobromophthalein	33.3 ± 3.9	58.5 ± 2.7†
Glutathione reductase [nmoles·min^{-1}·(mg protein)$^{-1}$]	140.4 ± 7.3	118.4 ± 5.6†

* Values were obtained from animals maintained for 6 weeks on either a vitamin A deficient or a vitamin A containing (control) diet (see Table 1). Each result is the mean ± S.E. of five rats.

† Significantly different from controls ($P < 0.05$ by Student's *t*-test).

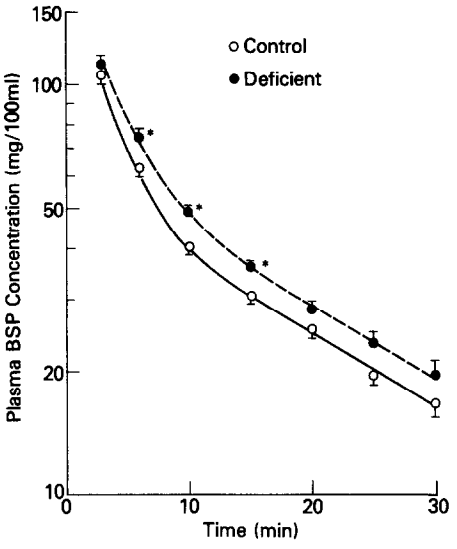

Fig. 1. Plasma disappearance of BSP in rats maintained for 6 weeks on a vitamin A deficient or a vitamin A containing (control) diet (see Table 1). BSP (100 mg/kg) was administered i.v. (femoral vein) as an aqeuous solution (2 ml/kg). Each point is the mean ± S.E. of six rats. An asterisk indicates that the value is significantly different from the control ($P < 0.05$ by Student's *t*-test).

deficient group so that growth rates were equivalent in the two groups. At 6 weeks, there were no significant differences in body or liver weights between the vitamin A deficient and control groups, although vitamin A was not detectable (< 0.5 μg/g) in the livers of the deficient animals (Table 1). All subsequent experiments were performed with animals that had received the appropriate diets for 6 weeks.

In vitro *enzyme activities and glutathione levels.* In vitamin A deficient rats, there was a significant decrease in hepatic glutathione reductase activity and significant increases in the GSH *S*-aryltransferase activities toward both DCNB and BSP (Table 2). The increase in transferase activity was greater with BSP as substrate than with DCNB (76 vs 41 per cent). No differences were found in the hepatic levels of GSH or total glutathione (GSH + GSSG) between the control and deficient animals.

Plasma disappearance, hepatic uptake and biliary excretion of BSP. Following an i.v. injection of BSP, the rate of disappearance of the drug from the plasma during the distribution (alpha) phase was significantly lower in vitamin A deficient animals (Fig. 1). Higher plasma concentrations of BSP were also noted in these animals with ligated bile ducts, and this correlated with lower hepatic content of the dye (Table 3). Thin-layer chromatographic analysis of the hepatic dye indicated that, at 15 and 45 min after

Table 3. Hepatic uptake of sulfobromophthalein (BSP) in vitamin A deficiency*

	Time after injection			
	15 min		45 min	
	Control	Deficient	Control	Deficient
Plasma BSP (mg/100 ml)	58.4 ± 2.0	72.2 ± 2.5†	49.3 ± 0.9	50.5 ± 1.0
Liver BSP (mg/g)	0.81 ± 0.06	0.55 ± 0.04†	0.43 ± 0.04	0.26 ± 0.02†
% Conjugated BSP in liver	78.7 ± 0.6	88.6 ± 1.7†	82.3 ± 1.0	90.8 ± 2.0†

* Animals were maintained for 6 weeks prior to the experiment on either a control or a vitamin A deficient diet (see Table 1). BSP (100 mg/kg; 2 ml/kg) was adminstered i.v., 60 min after bile duct ligation. Each result is the mean ± S.E. of six rats.

† Significantly different from controls ($P < 0.05$ by Student's *t*-test).

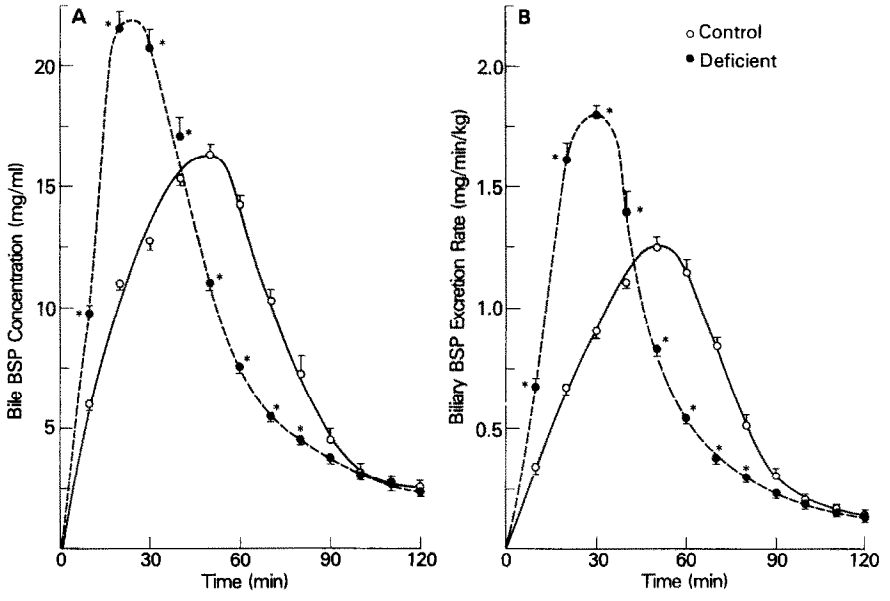

Fig. 2. Biliary concentration (A) and excretion rate (B) of BSP in rats maintained for 6 weeks on a vitamin A deficient or a vitamin A containing (control) diet (see Table 1). BSP (100 mg/kg) was administered i.v. (femoral vein) as an aqueous solution (2 ml/kg). Each point is the mean ± S.E. of six rats. An asterisk indicates that the value is significantly different from the control ($P < 0.05$ by Student's *t*-test).

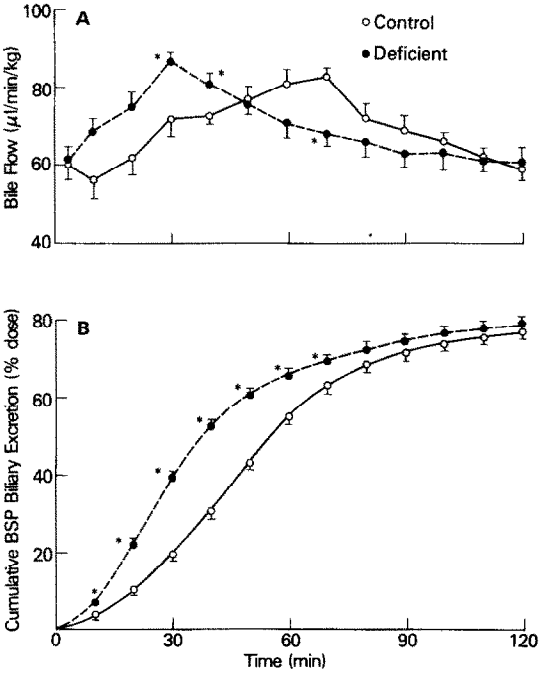

Fig. 3. Bile flow (A) and cumulative excretion (B) of BSP in rats maintained for 6 weeks on a vitamin A deficient or a vitamin A containing (control) diet (see Table 1). BSP (100 mg/kg) was administered i.v. (femoral vein) as an aqueous solution (2 ml/kg). Each point is the mean ± S.E. of six rats. An asterisk indicates that the value is significantly different from the control ($P < 0.05$ by Student's *t*-test).

BSP administration, vitamin A deficient animals had a greater proportion of the total BSP in the conjugated form (Table 3).

Plots of biliary BSP concentration vs time and biliary BSP excretion rate vs time indicated a more rapid and a greater excretion of BSP in vitamin A deficiency (Fig. 2). This was reflected in the enhanced cumulative biliary excretion of BSP in deficiency (Fig. 3B). Although the rates of bile flow in the control and deficient animals were similar at the start

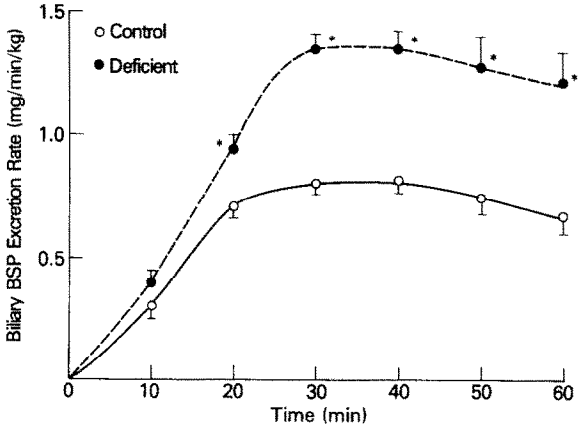

Fig. 4. Biliary excretion rate of BSP in rats maintained for 6 weeks on a vitamin A deficient or a vitamin A containing (control) diet (see Table 1). BSP was infused ($3\ mg \cdot min^{-1} \cdot kg^{-1}$) as an aqueous solution (1.67 ml/hr) via the femoral vein. Each point is the mean ± S.E. of six rats. An asterisk indicates that the value is significantly different from the control ($P < 0.05$ by Student's *t*-test).

Table 4. Effect of vitamin A deficiency on metabolism, biliary concentration, transport maximum (*Tm*) and hepatic storage of sulfobromophthalein (BSP)*

	Control	Deficient
Bile flow ($\mu l \cdot min^{-1} \cdot kg^{-1}$)		
Before infusion	59.2 ± 10.0	61.0 ± 4.4
At *Tm*	70.3 ± 4.4	76.9 ± 4.2
% Conjugated BSP in bile at *Tm*	88.4 ± 0.7	92.5 ± 0.4†
Bile BSP concentration at *Tm* (mg/ml)		
Total BSP	12.2 ± 0.5	17.6 ± 1.0†
Free BSP	1.4 ± 0.1	1.3 ± 0.1
Conjugated BSP	10.8 ± 0.4	16.3 ± 0.9†
Transport maximum, *Tm* ($mg \cdot min^{-1} \cdot kg^{-1}$)	0.85 ± 0.03	1.38 ± 0.08†
Contribution to *Tm* of free BSP ($mg \cdot min^{-1} \cdot kg^{-1}$)	0.097 ± 0.004	0.102 ± 0.007
Contribution to *Tm* of conj. BSP ($mg \cdot min^{-1} \cdot kg^{-1}$)	0.76 ± 0.03	1.27 ± 0.08†
Hepatic storage‡		
(mg BSP) · (mg BSP/100 ml plasma)$^{-1}$ · (kg body wt)$^{-1}$	0.88 ± 0.04	0.64 ± 0.05†

* BSP was infused (3 $mg \cdot min^{-1} \cdot kg^{-1}$) as an aqueous solution (1.67 ml/hr) via the femoral vein in rats maintained for 6 weeks on either a vitamin A deficient or a vitamin A containing (control) diet (see Table 1). Each result is the mean ± S.E. of six rats.

† Significantly different from controls ($P < 0.05$ by Student's *t*-test).

‡ Defined as the amount of BSP in the liver divided by the plasma BSP concentration [15].

of the experiment, the choleretic effect of BSP paralleled the biliary excretion rate of the dye, thus resulting in significant differences in bile flow rate at the 30-, 40- and 70-min time points (Fig. 3A).

Rats were infused continuously with BSP to determine the transport maximum (*Tm*) and hepatic storage of the dye. Maximal excretion (*Tm*) of BSP was reached about 30 min after the start of infusion (Fig. 4). In vitamin A deficient animals, the *Tm* was increased significantly (Fig. 4, Table 4). Analysis of the bile obtained between 30 and 40 min (that is, at *Tm*) indicated that the concentration of free BSP, and, thus, the contribution to *Tm* of this component, was similar in the two groups. The biliary concentration and the contribution to *Tm* of the conjugated BSP, however, were increased in deficiency (Table 4). These changes were accompanied by a concomitant decrease in hepatic storage of BSP.

DISCUSSION

We have demonstrated in the present communication that hepatic GSH *S*-aryltransferase activity is increased in vitamin A deficiency. Since GSH *S*-aryltransferase catalyzes the reactions between GSH and aromatic compounds containing activated halogen or nitro groups [20], it is not surprising that increased activities were seen toward both DCNB and BSP in the deficient animals. It is interesting, however, that a greater increase in activity was seen with BSP than with DCNB. It is possible that different transferases catalyze the conjugation with GSH of BSP and DCNB. In this regard, it has been shown that norethandrolone slightly inhibits the conjugation of DCNB [21] but stimulates the conjugation of BSP [22] with GSH. The increase in GSH *S*-aryltransferase activity was not due to a change in the GSH:GSSG ratio, which has been shown to be important in regulating the activity of several enzymes [23]. Since the hepatic glutathione reductase activity was decreased in vitamin A deficiency without a corresponding increase in the GSSG level, it is probable that glutathione reductase is not a rate-limiting enzyme.

The effects of increased enzymatic BSP conjugation *in vitro* on BSP plasma clearance and biliary excretion have been examined to determine whether BSP conjugation with GSH was rate-limiting in BSP elimination *in vivo*. Studies by Whelan *et al.* [3], Varga *et al.* [4] and Zsigmond and Solymoss [24] indicate that conjugation of BSP facilitates dye transport into bile and is the rate-limiting step in the transfer *in vivo* of BSP from blood to bile. Boyland and Grover [21], however, compared BSP retention with human liver GSH *S*-aryltransferase activity and concluded that BSP conjugation is not the rate-limiting step in hepatic dye clearance. A similar conclusion was reported by Klaassen and Plaa [6], who demonstrated that the increased BSP excretion in bile of phenobarbital-treated rats is not due to an increased BSP conjugation but rather to an increase in bile flow. In the present study, a much greater increase in BSP conjugation *in vitro* occurred in vitamin A deficient rats than occurred in phenobarbital-treated animals [6]. In addition, a greater hepatic content and biliary concentration of conjugated BSP in deficiency was demonstrated *in vivo*. If conjugation of BSP is the rate-limiting step in its biliary excretion, an increased enzymatic rate of conjugation should result in an increase in the excretion of the dye. This has been clearly demonstrated in the present study with vitamin A deficient rats. Interestingly, the increase (76 per cent) in the *in vitro* conjugation rate of BSP (Table 2) was similar to the increase (62 per cent) in *Tm* (Table 4) seen in vitamin A deficiency. Furthermore, the increase in *Tm* was due entirely to the increase in the proportion of conjugated BSP appearing in the bile.

The initial, slower rate of disappearance of BSP from the plasma seen in vitamin A deficiency was probably due to the reduction in hepatic uptake of

the dye, and this may have been a result of impaired storage capacity of these livers. Whether this reduction in hepatic storage of BSP is due to a reduction in its binding or storage sites, or to other mechanisms, is presently not known.

These results demonstrate that in vitamin A deficient rats conjugation of BSP *in vitro* and *in vivo* is increased, resulting in an enhanced biliary excretion of the dye; this suggests that conjugation with GSH is a rate-limiting step in the biliary excretion of BSP. Further studies are needed to determine whether such an effect of vitamin A deficiency can protect rats from toxic chemicals that are metabolized by the glutathione *S*-transferases and excreted in the bile.

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REFERENCES

1. C. D. Klaassen, *CRC Crit. Rev. Toxic.* **4**, 1 (1975).
2. B. Combes, *J. clin. Invest.* **44**, 1214 (1965).
3. G. Whelan, J. Hoch and B. Combes, *J. Lab. clin. Med.* **75**, 542 (1970).
4. F. Varga, E. Fischer and T. S. Szily, *Biochem. Pharmac.* **23**, 2617 (1974).
5. B. G. Priestly and G. L. Plaa, *Proc. Soc. exp. Biol. Med.* **132**, 881 (1969).
6. C. D. Klaassen and G. L. Plaa, *J. Pharmac. exp. Ther.* **161**, 361 (1968).
7. T. E. Gram, C. L. Litterst and E. G. Mimnaugh, *Drug. Metab. Dispos.* **2**, 254 (1974).
8. P. L. Grover and P. Sims, *Biochem. J.* **90**, 603 (1964).
9. J. Goldstein and B. Combes, *J. Lab. clin. Med.* **67**, 863 (1966).
10. C. E. Mize and R. G. Langdon, *J. biol. Chem.* **237**, 1589 (1962).
11. G. L. Ellman, *Archs. Biochem. Biophys.* **82**, 70 (1959).
12. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
13. R. E. Dugan, N. A. Frigerio and J. M. Siebert, *Analyt. Chem.* **36**, 114 (1964).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. C. D. Klaassen and G. L. Plaa, *J. appl. Physiol.* **22**, 1151 (1967).
16. F. E. D'Amour, F. R. Blood and D. A. Belden, Jr., *Manual for Laboratory Work in Mammalian Physiology*, 3rd Edn., Expt. 3. University of Chicago Press, Chicago (1969).
17. T. Iga, Y. Sugiyama, M. Yokoto, Y. Tomono, S. Awazu and M. Hanano, *Biochem. Pharmac.* **26**, 1867 (1977).
18. P-J. Schulze and G. Czok, *Toxic. appl. Pharmac.* **32**, 213 (1975).
19. F. J. Whelan and G. L. Plaa, *Toxic. appl. Pharmac.* **5**, 457 (1963).
20. L. F. Chasseaud, *Drug Metab. Rev.* **2**, 185 (1973).
21. E. Boyland and P. L. Grover, *Clinica. chim. Acta* **16**, 205 (1967).
22. J. Goldstein and B. Combes, *J. Lab. clin. Med.* **67**, 830 (1966).
23. N. S. Kosower and E. M. Kosower, in *Glutathione: Metabolism and Function* (Eds. I. M. Arias and W. B. Jakoby), p. 159. Raven Press, New York (1976).
24. G. Zsigmond and B. Solymoss, *J. Pharmac. exp. Ther.* **183**, 499 (1972).