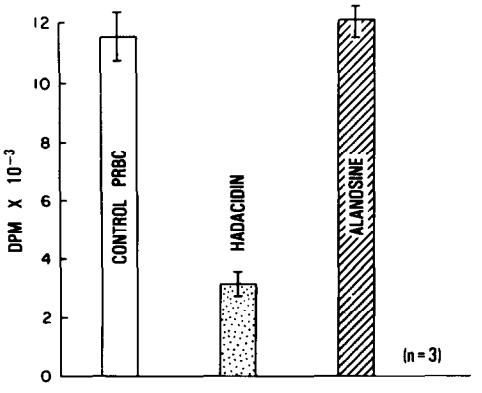

Fig. 1. Effects of adenylosuccinate synthetase inhibitors on incorporation of [^{3}H]hypoxanthine into the acid-insoluble fraction (nucleic acids) of malaria infected erythrocytes. Malaria cultures (4.8% PRBC) were incubated (3 hr) with and without inhibitors (5×10^{-5} M). Unparasitized, untreated RBC cultures served as controls. Following incubation, PCA extracts were prepared on all cultures. The acid-insoluble precipitate was recovered by centrifugation, washed three times in cold PCA, and solubilized in 1 N NaOH. Aliquots were taken for counting in a liquid scintillation counter. These data provide an estimate of incorporation of [^{3}H]hypoxanthine into parasite nucleic acids. Control RBC cultures contained 496 ± dpm, N = 3.

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a unique metabolic target for the design of new chemotherapy.

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REFERENCES

1. H. K. Webster and J. M. Whaun, *Prog. clin. biol. Res.* **55**, 557 (1981).
2. H. K. Webster and J. M. Whaun, *J. clin. Invest.* **70**, 461 (1982).
3. P. Reyes, P. K. Rathod, D. J. Sanchez, J. E. Mrema, K. H. Rieckmann and H. G. Heidrick, *Molec. biochem. Parasit.* **5**, 275 (1982).
4. I. W. Sherman, *Microbiol. Rev.* **43**, 453 (1979).
5. G. J. Brewer, in *The Red Blood Cell* (Ed. D. M. Surgenor), p. 473. Academic Press, New York (1975).
6. B. Lowry, M. Williams and L. London, *J. biol. Chem.* **237**, 1622 (1962).
7. M. Lave, B. Tip and F. Rudolph, *Biochem. Pharmac.* **26**, 1353 (1977).
8. W. Trager and J. B. Jensen, *Science* **193**, 673 (1976).
9. J. D. Haynes, C. L. Diggs, F. A. Hines and R. E. Desjardins, *Nature, Lond.* **263**, 767 (1976).
10. H. K. Webster and J. M. Whaun, *J. Chromat.* **209**, 283 (1981).
11. A. Tyagi and D. A. Cooney, *Cancer Res.* **40**, 4390 (1980).

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Substrate induction of the biliary excretion of sulfobromophthalein in rats

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Renal organic acid transport (as measured by *p*-aminohippurate uptake into renal cortical slices and isolated tubuli) is stimulated by penicillin pretreatment [1, 2]. Intestinal glucose absorption is also induced by prolonged infusion of glucose, which is thought to increase the number of carriers in the basolateral membranes of enterocytes [3, 4]. In rats, repeated oral administration of taurocholate [5] and cholate [6] enhanced the biliary excretion of taurocholate due to an increase in the density of bile acid carriers in the liver surface membrane [6], but did not affect the biliary excretion of two xenobiotics, phenol-3,6-dibromphthalein and ouabain [5]. All these observations suggest that it is possible to induce various transport processes by prolonged administration of their substrates, and that the increase in overall transcellular transport is due to increased carrier-protein synthesis.

The present experiments were designed to study the inducibility of the biliary excretory system for foreign cholephilic organic acids. Sulfobromophthalein (BSP), BSP-glutathione (BSP-GSH), rose bengal and eosine, which are thought to be excreted into bile by the same transport mechanism, were used as inducers [7]. BSP is known to be conjugated with glutathione (GSH) in the liver whereas rose bengal and eosine are excreted into bile unchanged. Of the four compounds, only BSP proved to be an inducer. In this paper, experimental data which show that increased biliary excretion of BSP is due to increased conjugation of BSP with GSH rather than enhancement of the hepatic transport function itself are presented.

Materials and methods. Disodium salt of BSP was purchased from Merck A.G. (Darmstadt, F.R.G.); rose bengal from Fluka A.G. (Buchs, Switzerland); water-soluble eosine (C.I. 45380) from Reanal (Budapest, Hungary); and diethyl maleate from Eastman Chemicals (Rochester). BSP-GSH was synthesized from BSP by the method of Whelan *et al.* [8].

The experiments were performed on male Sprague–Dawley rats weighing 200–250 g. The animals were pretreated with BSP (60 and 120 μmole/kg i.p.), BSP-GSH (80 μmole/kg i.p.), rose bengal (50 μmole/kg i.p.) and eosine

(150 μmole/kg i.p.) twice daily for 2 days. The biliary excretion of cholephils was investigated 18 hr after the last injection, when no dye could be detected in the bile. Under urethane (1.2 g/kg i.p.) anaesthesia, a median laparotomy was performed, the bile duct was cannulated with PE-10 polyethylene tubing, and bile was collected 20 min prior to i.v. injection of the dye to be investigated (basal bile flow), and every 20 min for 1 hr after injection. The body temperature of the rats was maintained at 37° by means of a heat lamp. The livers were weighed at the end of the experiments. When the biliary excretion of unconjugated BSP was being studied, diethyl maleate (DEM; 0.7 ml/kg i.p., 30 min prior to the BSP injection) was employed to prevent hepatic conjugation of BSP with GSH. DEM decreases the GSH concentration in liver to 5–10% of the respective control value in 30 min [9].

Bile concentrations of the drugs were determined spectrophotometrically after an appropriate dilution of the bile samples. Unconjugated BSP was separated from BSP-GSH on Whatman No. 1 filter paper using an ascending system consisting of *n*-butanol–acetic acid–water (4:1:2, v/v). The paper was exposed to ammonia vapour, the spots were cut out and the drug was eluted and measured spectrophotometrically. The activity of glutathione *S*-transferase towards BSP was measured by the method of Habig *et al.* [10]. GSH concentration in the liver was determined by the method of Sedlak and Lindsay [11] as described by Wong and Klaassen [12].

The biliary excretion of the drugs was calculated as a product of bile flow and biliary concentration. The data in the tables and text represent means ± standard errors (S.E.). Significance was calculated by the *t*-test.

Results. Pretreatment with BSP at a dose of 60 μmole/kg i.p. twice daily for 2 days proved to be sufficient to increase the biliary excretion of total BSP (Table 1). The increase was mainly due to the enhanced excretion of BSP-GSH. Basal bile flow and liver weight of the pretreated rats did not differ significantly from those of the control group. After pretreatment with BSP-GSH, rose bengal and eosine, the biliary excretion of total BSP was unchanged.

Pretreatment with BSP or BSP-GSH failed to influence either the biliary excretion of BSP when its conjugation with GSH was prevented by DEM, or the excretion of i.v. administered BSP-GSH (Table 2).

Pretreatment with BSP (60 μmole/kg i.p., twice daily for 2 days) did not affect the biliary excretion of rose bengal (50 μmole/kg i.v.; controls: 163 ± 13.5 nmole/min per kg, $n = 6$; treated group: 168 ± 17.3 nmole/min per kg, $n = 6$) or eosine (150 μmole/kg i.v.; controls: 810 ± 88.7 nmole/min per kg, $n = 8$; treated group: 873 ± 79.8 nmole/min per kg, $n = 8$).

The activity of hepatic glutathione *S*-transferase towards BSP was significantly increased by BSP pretreatment (Table 3). The GSH content of liver was also elevated in BSP-pretreated rats. In contrast, both the GSH-transferase activity and the GSH concentration were unchanged fol-

Table 1. Effect of pretreatment with cholephilic organic acids on liver weight, basal bile flow and biliary excretion of total BSP

Pretreatment	Dose* (μmole/kg i.p.)	Liver weight (g/kg)	Basal bile flow (μl/min per kg)	Biliary excretion of total BSP† (nmole/min per kg)	Conjugated BSP in bile (%)
Saline		32.5 ± 0.89	72.6 ± 8.48	443 ± 34.8	76.2 ± 1.30
BSP	60	31.0 ± 1.12	81.5 ± 7.32	600 ± 48.2‡	86.7 ± 1.15‡
	120	32.2 ± 0.47	81.0 ± 10.1	660 ± 54.8‡	86.0 ± 1.18‡
BSP-GSH	80	32.7 ± 0.81	78.3 ± 9.05	485 ± 59.6	78.0 ± 1.78
Rose bengal	50	31.3 ± 0.84	76.8 ± 5.93	473 ± 67.5	79.3 ± 1.65
Eosine	150	31.5 ± 0.81	70.6 ± 2.88	453 ± 59.8	79.7 ± 1.92

* Rats were pretreated intraperitoneally with a 0.9% solution of NaCl (5 ml/kg) and with cholephilic organic acids at the doses indicated in the table twice daily for 2 days.

† BSP (120 μmole/kg i.v.) was given 18 hr after the last dose of pretreatment and total (unconjugated plus conjugated) BSP excretion was measured from bile collected for 60 min.

‡ Significant difference from control group ($P < 0.05$).

The data represent mean values ± S.E. of 8–14 rats.

Table 2. Effect of pretreatment with BSP and BSP-GSH on the biliary excretion of BSP-GSH in rats and of unconjugated BSP in GSH-depleted rats

	Biliary excretion (nmole/min per kg) BSP (80 μmole/kg i.v.)*			BSP-GSH (80 μmole/kg i.v.)†		
Pretreatment	0–20 min	20–40 min	40–60 min	0–20 min	20–40 min	40–60 min
Saline	373 ± 42.3	475 ± 68.8	341 ± 53.4	1306 ± 81.4	448 ± 40.0	149 ± 11.5
BSP	331 ± 29.0	387 ± 34.3	374 ± 32.5	1205 ± 94.2	507 ± 53.6	172 ± 18.3
BSP-GSH	379 ± 57.7	463 ± 32.3	320 ± 23.0	1198 ± 97.6	434 ± 49.2	153 ± 15.2

Rats were pretreated with BSP (120 μmole/kg i.p.), BSP-GSH (80 μmole/kg i.v.) and a 0.9% solution of NaCl (5 ml/kg i.p.) twice daily for 2 days. Biliary excretion of unconjugated BSP and BSP-GSH was investigated following the i.v. administration of drugs 18 hr after the last pretreatment.

* When the biliary excretion of unconjugated BSP was being investigated, 0.7 ml/kg diethyl maleate was injected i.p. 30 min prior to the i.v. administration of BSP in order to prevent the conjugation of BSP with GSH.

‡ BSP-GSH was synthesized *in vitro* [8].

The data represent mean values ± S.E. of 4–9 rats.

Table 3. Effect of BSP, rose bengal and eosine on the glutathione *S*-transferase activity towards BSP and the glutathione (GSH) concentration in rat liver

Pretreatment	GSH-transferase activity (nmole/min per g liver)	GSH concentration (μmole/g liver)
Saline	1056 ± 47	8.55 ± 0.39
BSP	1480 ± 100*	11.3 ± 0.52*
Rose bengal	1156 ± 156	7.52 ± 0.56
Eosine	986 ± 102	10.3 ± 0.91

Rats were pretreated with sulfobromophthalein (BSP, 120 μmole/kg i.p.), rose bengal (50 μmole/kg i.p.), eosine (150 μmole/kg i.p.) and a 0.9% solution of NaCl (5 ml/kg i.p.) twice daily for 2 days. The enzymic activity and GSH concentration in the liver were determined 18 hr after the last injection.

The data represent mean values ± S.E. of 8–10 (pretreated groups) or 24 (control group) rats.

* Significant difference from control group ($P < 0.05$).

lowing pretreatment with rose bengal and eosine.

Discussion. Increased liver mass and bile flow may enhance the biliary excretion of various cholephilic organic acids as has been demonstrated, for example, in phenobarbital-treated rats [13, 14], however, pretreatment with BSP, BSP-GSH, rose bengal and eosine failed to influence these parameters (Table 1). Although BSP, rose bengal and eosine seem to be transported from the blood to the bile by the same mechanism [7], pretreatment with BSP did not affect the biliary excretion of rose bengal and eosine, and pretreatment with eosine or rose bengal did not influence the biliary excretion of BSP. These findings lend support to the conclusion that BSP does not induce hepatobiliary transport itself.

The enhanced biliary output of total BSP after BSP pretreatment was solely due to the increased excretion of BSP-GSH. The excretion rate of the parent compound (unconjugated BSP) was not affected even when its conjugation was prevented by DEM (Table 2). Since both the activity of glutathione *S*-transferase and the GSH content of liver were increased by BSP treatment, the enhanced biliary output of total BSP may be explained by stimulation of the conjugation of BSP with GSH. Conjugation of BSP with GSH facilitates the biliary excretion of BSP because BSP-GSH is excreted into the bile at a faster rate than unconjugated BSP [8, 15, 16], and the conjugation eliminates the parent compound which is a strong inhibitor of BSP-GSH excretion [17]. The increased GSH content of liver, which may also enhance the conjugation, may be regarded as a compensatory elevated synthesis of GSH caused by enhanced consumption of GSH by BSP during the pretreatment.

A hepatic cytoplasmic protein, ligandin, binds various cholephilic organic acids such as bilirubin, indocyanine green and BSP [18]. In pioneer investigations, it was proposed that ligandin might facilitate the transport of BSP [19] and bilirubin [20] from the blood to the bile; however, some recent findings do not support this view [21–24]. It has been emphasized that ligandin, shown to be identical to glutathione *S*-transferase [25], may play a more important role in the overall hepatic transport of BSP as an enzyme rather than as an intracellular binding and/or carrier protein [24]. Our results support this view.

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REFERENCES

1. G. H. Hirsch and J. B. Hook, *J. Pharmac. exp. Ther.* **174**, 152 (1970).
2. D. G. Pegg, J. Bernstein and J. B. Hook, *Proc. Soc. exp. Biol. Med.* **151**, 720 (1976).
3. T. Z. Csáky and E. Fischer, *Experientia* **33**, 223 (1977).
4. T. Z. Csáky and E. Fischer, *Diabetes* **30**, 568 (1981).
5. J. B. Watkins and C. D. Klaassen, *J. Pharmac. exp. Ther.* **218**, 181 (1981).
6. R. R. Simon, E. M. Sutherland and M. Gonzales, *J. clin. Invest.* **70**, 401 (1982).
7. E. Fischer and F. Varga, *Archs int. Pharmacodyn.* **209**, 349 (1974).
8. G. Whelan, J. Hock and B. Combes, *J. Lab. clin. Med.* **75**, 542 (1970).
9. E. Boyland and L. F. Chasseaud, *Biochem. Pharmac.* **19**, 1526 (1970).
10. W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. biol. Chem.* **249**, 7130 (1974).
11. J. Sedlak and R. H. Lindsay, *Analyt. Biochem.* **25**, 192 (1968).
12. K. L. Wong and C. D. Klaassen, *Toxicology* **19**, 39 (1981).
13. C. D. Klaassen and G. L. Plaa, *J. Pharmac. exp. Ther.* **161**, 361 (1968).
14. C. D. Klaassen, *Biochem. Pharmac.* **19**, 1241 (1970).
15. G. Whelan and B. Combes, *J. Lab. clin. Med.* **78**, 230 (1971).
16. P. J. Schulze, G. Czok and H. U. Brock, *Naunyn-Schmiedeberg's Archs Pharmac.* **296**, 79 (1976).
17. Z. Gregus, E. Fischer and F. Varga, *Biochem. Pharmac.* **26**, 1951 (1977).
18. A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. clin. Invest.* **48**, 2156 (1969).
19. H. Reyes, A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. clin. Invest.* **50**, 2242 (1971).
20. E. Tipping and B. Ketterer, in *Transport by Proteins* (Eds. G. Blauer and H. Sund), pp. 369–383. W. de Gruijter, Berlin (1978).
21. D. K. F. Meijer, R. J. Vonk, K. Keulcmans and J. G. Weitering, *J. Pharmac. exp. Ther.* **202**, 8 (1977).
22. C. D. Klaassen, *J. Pharmac. exp. Ther.* **195**, 311 (1975).
23. D. K. F. Meijer, A. Blom and J. G. Weitering, *Biochem. Pharmac.* **31**, 2539 (1982).
24. Z. Gregus and C. D. Klaassen, *J. Pharmac. exp. Ther.* **221**, 242 (1982).
25. W. H. Habig, M. F. Pabst, G. Fleischner, Z. Gatmaitan, I. M. Arias and W. B. Jakoby, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3879 (1974).