Results and discussion. The results in Table I indicate that the preparations of LRF of bovine and ovine origin were active in depleting ovarian ascorbic acid in immature rats pretreated with gonadotropin, and in elevating plasma LH in chronically ovariectomized estrogen progesterone-treated rats. These two tests show that LRF preparations were clearly effective in releasing LH *in vivo*, at doses of 125-1300 μg. The same preparations were also significantly active *in vitro* in releasing LH from the isolated rat anterior pituitary tissue. Doses of 600-800 μg of purified LRF increased LH release *in vitro* 2- or 3-fold, which is in agreement with results reported previously (10). Thus a dose of 800 μg of CY-2-B-6 stimulated LH release by 204% (95% fiducial limits of the assay 140-290%) and the variance ratio F between stimulated and unstimulated samples was highly significant at 13.3.

Addition of these active LRF preparations to incubation media did not inhibit prolactin secretion by the isolated rat pituitary. In some cases the doses tested for inhibition of prolactin secretion were 10 times larger than doses found active in the LRF tests.

The results suggested that the LRF and PIF activity are not shared by the same molecule. Other data supporting this concept comes from physiological experiments and observations(1).

Summary. Preparations of hypothalamic luteinizing hormone releasing factor (LRF) of bovine and ovine origin, found active on the basis of *in vivo* as well as *in vitro* tests, did not have an inhibitory effect on pituitary prolactin release *in vitro*. The results indicate that LRF and prolactin inhibiting factor (PIF) are not the same.

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Phenol 3, 6 Dibromphthalein Disulfonate, A New Compound for the Study of Liver Disease. (29550)

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Conjugation of BSP (phenoltetrabromphthalein disulfonate, sulfobromophthalein) with glutathione(1,2) represents the major pathway in the transfer of BSP from plasma to bile. However, the role of conjugation in affecting the rate of removal of BSP from plasma or excretion from the liver cell into bile has not been determined. As a result of studies concerning the conjugation and excre-

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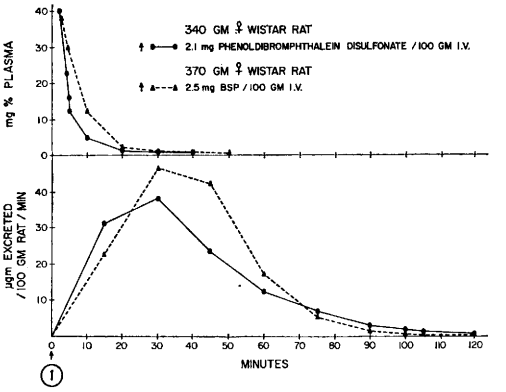
tion of compounds related in structure to BSP(3), a new compound, phenol 3,6 dibromphthalein disulfonate, has been prepared and found to be removed from plasma and recovered mostly in the bile. During the transfer from plasma to bile the new compound is not conjugated. The close similarity in structure between the new compound and BSP suggests that the new compound will be useful in man and animal in distinguishing factors affecting uptake and excretion from those affecting conjugation.

Materials and methods. Phenol 3,6 dibromphthalein disulfonate (mol wt 680 Na salt) was prepared by Dr. Lloyd Felton, Hynson, Westcott and Dunning, Baltimore, Md. The compound is made by condensation of crystalline 3,6 dibromphthalic anhydride (4) with phenol followed by a sulfonation procedure.

Sulfur analysis of the compound (Calc. S, 9.41% Found S, 8.84%) shows it to be a disulfonate which is further substantiated by the finding that the compound has the same electrophoretic migration as BSP. Paper electrophoresis (5 N acetic acid, pH 1.9, 500 v, 3-4 hours, Series B Durrum cell) and paper chromatography (Whatman #4 paper, descending, n-propanol:acetic acid:H_2O;10:1:5) were used for detection of derivatives. BSP and phenoldibromphthalein disulfonate were quantitatively estimated at 574 mμ using the method of Seligson, Marino and Dodson(5). Glutathione conjugating enzyme was prepared according to Booth, Boyland and Sims(6) except for the substitution of phosphate buffers for pyrophosphate buffers.

Phenol 3,6 dibromphthalein disulfonate and BSP dissolved in 0.9% saline (6 μmoles/ml) were administered by intravenous injection (3 μmoles to 6 μmoles/100 g body weight) in equimolar amounts to Wistar rats and albino rabbits. Wistar rats (250-350 g) were anesthetized with intraperitoneal pentobarbital (5 mg/100 g) and bile collected from a polyethylene catheter inserted into the common duct. Blood samples were obtained from the tail vein. Rabbits received local xylocaine anesthesia in a hind limb. A PE 50 catheter was passed into the inferior vena cava through a leg vein. Blood samples were collected into heparinized syringes. Samples showing hemolysis were discarded.



COMPARISON OF PLASMA DISAPPEARANCE OF BSP AND PHENOLDIBROMPHTHALEIN DISULFONATE

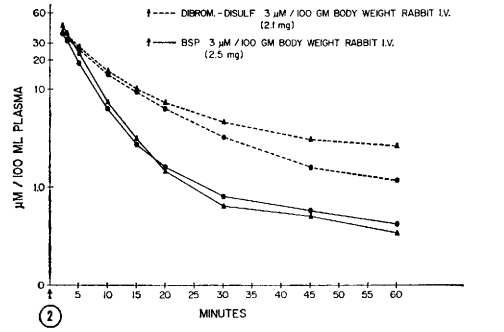


FIG. 1. Disappearance from plasma and excretion rate in bile of phenol 3,6 dibromphthalein disulfonate and BSP. Equimolar amounts of the compounds were injected into rats and blood samples obtained from the tail vein. Disappearance from plasma of both compounds is apparently quite similar but much of the BSP is conjugated. Therefore the disappearance rate of injected BSP is not known but must be greater than shown.

FIG. 2. Disappearance from plasma of phenol 3,6 dibromphthalein disulfonate and BSP after equimolar injection into rabbits. Each rabbit received an injection of each compound at 2-hour interval. Order of injection was alternated. The disappearance rate of phenol 3,6 dibromphthalein disulfonate is less than that obtained by plotting total BSP. Since BSP conjugate may also be present in plasma the disappearance rate of injected BSP may be greater.

Results. Eight rats received injections of phenoldibromphthalein disulfonate and/or BSP. Disappearance rate of BSP from plasma, uncorrected for circulating BSP conjugates, was similar to that of the new compound (Fig. 1).

Because of the inaccuracies inherent in the

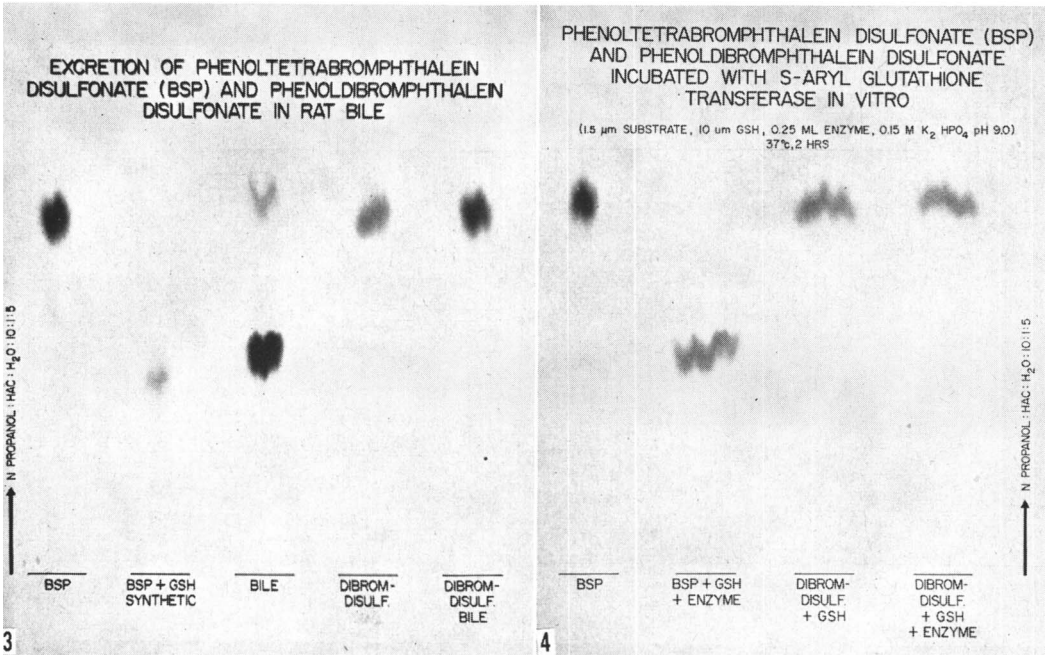


FIG. 3. Chromatogram of phenol 3,6 dibromphthalein disulfonate and BSP and derivatives present in bile. BSP and phenol 3,6 dibromphthalein disulfonate (Dibrom-Disulf) have the same Rf in n-propanol:acetic acid:H_2O:10:1:5). Center column is bile obtained after injection of BSP and shows that the main component is BSP-glutathione comparable to that made synthetically, shown in the adjacent column. Bile obtained after injection of phenol 3,6 dibromphthalein disulfonate Dibrom. Disulf. Bile) shows that there is no apparent alteration of the compound.

FIG. 4. Incubation of phenol 3,6 dibromphthalein disulfonate and BSP with glutathione-conjugating enzyme *in vitro*. BSP in presence of glutathione and enzyme is converted completely to a conjugate. Phenol 3,6 dibromphthalein disulfonate (dibrom-disulf) does not react with glutathione, even in the presence of the enzyme.

collection of blood from the tail vein, especially for comparison purposes, a larger animal was chosen. Each rabbit received an injection of BSP and the new compound at a 2-hour interval. The order of administration was alternated. Regardless of order of administration, the disappearance rate of the phenoldibromphthalein disulfonate was less than BSP (Fig. 2).

The rate of excretion of the 2 compounds into bile was also similar (Fig. 1). Mean recovery of the injected dose by the end of 2 hours was 83% for the new compound and 87% for BSP.

Paper chromatography (Fig. 3) and paper electrophoresis of bile revealed the previously described patterns of the BSP derivatives(1). In contrast, the Rf and electrophoretic mobility of the phenoldibromphthalein disulfonate recovered in bile was identical to that administered (Fig. 3) and did not react with ninhydrin.† Analysis of bile obtained from an anesthetized rabbit after injection of each compound revealed no evidence of alteration of injected phenoldibromphthalein disulfonate.

Attempts were made to prepare a synthetic phenol 3,6 dibromphthalein disulfonate-glutathione conjugate. Equimolar amounts (80 μmoles/2 ml H_2O) of each compound were mixed and titrated to pH 8.0 with 0.1 N NaOH and incubated for 24 hours at 37°C. During this time the pH of the reaction mixture remained unchanged and chromatographic analysis did not reveal a glutathione conjugate. Similar preparations using BSP regularly results in the formation of a conju-

† An occasional trace of another derivative is seen and may represent a glutathione conjugate of some trace contaminant of phenol 3,6 dibromphthalein disulfonate.

gate associated with a fall in pH of the reaction mixture(1).

BSP or phenoldibromphthalein disulfonate (1.5 μmoles) was incubated with glutathione (10 μmoles) and 0.2 ml of glutathione conjugating enzyme in 3.0 ml of 0.15 M K_2HPO_4, pH 9.0 for 2 hours. Complete conjugation of BSP occurred (Fig. 4) but conjugation of phenol 3,6 dibromphthalein did not occur.

Discussion. The studies demonstrate that phenol 3,6 dibromphthalein disulfonate is removed from plasma and excreted in high concentration in bile, without conjugation. Conjugation of phenoltetrabromphthalein disulfonate (BSP) occurs *via* replacement of one of the bromine atoms(1,2). Apparently then, the two bromine atoms in the new compound are not subject to significant replacement by glutathione either enzymatically or non-enzymatically under the conditions studied.

The disappearance rate of phenol 3,6 dibromphthalein disulfonate from plasma is slower than BSP in the rabbit. A similar relationship, although not immediately apparent, may occur in the rat. Krebs(7) has shown by pooling blood samples of several rats, that 15 minutes following injection of BSP more than 50% of the total BSP color in plasma is attributable to circulating conjugates. Therefore the rate of disappearance of injected BSP is much more rapid than reflected by estimation of total BSP. Quantitative estimation of BSP conjugates by chromatographic methods is not technically feasible for the small amounts of plasma obtained. Therefore the true disappearance rate of unconjugated BSP is not known.

Schmid and Hammaker(8) have shown that bilirubin is bound more tightly to human serum albumin than to rat serum albumin. Differences in binding may affect the rate of removal of compounds from plasma. It is therefore not possible to predict the relative disappearance rates of the two compounds from human plasma.

The compounds differ by only 2 bromine atoms. It is possible, however, that this difference, and not conjugation, accounts for the different removal rates from rabbit plasma. However, regardless of the cause for their differences in removal from plasma, use of both compounds in a study of liver disease may clearly distinguish factors affecting uptake and excretion from those affecting conjugation.

Summary. Phenol 3, 6 dibromphthalein disulfonate has been prepared and its fate determined *in vivo*. After intravenous injection into rabbits it is removed from plasma at rates less than BSP. In the rat a similar but less apparent relationship may exist. Analysis of bile revealed that, unlike BSP, the new compound is not conjugated *in vivo*, nor was it possible to synthesize a glutathione conjugate *in vitro*. The new compound is useful for studies of hepatic uptake and excretion.

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