cation), although muscles of the ulnar group all exceed 5 cm in length. Adrian (3) found tenuissimus muscle fiber lengths of 13–23.5 mm with a mean of 17.3 mm for 21 intact fibers from a 12-cm long muscle; the longest fiber isolated from any muscle was 27 mm.

End-plates generally lie in the midportion of muscle fibers (5). The present results from electrical recording and by staining (Table I) agree by showing that end-plates are located predominantly in the middle third of intact fibers.

Summary. Single motor nerve fibers to bat web muscles were stimulated *in vivo,* and both ends of single muscle fibers were located by the presence of monophasic action potentials. The mean length of 12 such muscle fibers was 10.1 mm. Electrical responses of minimum latency were recorded in 6 of these fibers, demonstrating end-plate loci within the middle two quarters of muscle fibers, generally close to the middle. In addition, 71 muscle fibers were isolated after acid treatment. Mean length of 57 intact fibers was 14.1 mm and of 14 fibers with missing tips was 10.1 mm. The 14 fibers with missing tips and one intact fiber were stained for "lead-reactive substance" to locate their end-plates, which were within the middle two quarters of the fibers.

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Biliary Excretion of Phenol 3,6-Dibromphthalein Disulfonate in Rats Fed a Protein-Free Diet* (32802)

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It is generally accepted that much of the BSP (sulfobromophthalein sodium, phenoltetrabromphthalein disulfonate) taken up by liver cells undergoes conjugation with glutathione, and that conjugated BSP compounds account for most but not all of the dye excreted into bile of man, rats, and dogs (1–3). Whereas conjugation does not appear to affect hepatic uptake of BSP (4,5), evidence has been adduced that conjugation facilitates biliary excretion of the dye (4), and is an important determinant of the maximal rate at which BSP is transported from liver cells into bile (5). In the latter studies (5), feeding rats a protein-free diet for 2 days resulted in a decrease in the components of the hepatic BSP–glutathione conjugating system, with a 70% decrease in hepatic glutathione content, and a 25% fall in BSP–glutathione conjugating enzyme activity. When doses of BSP of 7.5 mg/100 gm of body weight and higher were administered intravenously to these animals, excretion of BSP into bile was diminished due to decreased excretion of conjugated dye. Biliary excretion of conjugated and total BSP was restored to control values, however, when hepatic glutathione content was maintained near normal levels by feeding a protein-free diet supplemented with 1% cystine.

The recent demonstration by Javitt (6), that phenol 3,6–dibromphthalein disulfonate, (diBSP) a compound related structurally to BSP, is readily excreted into bile of rats and

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rabbits as the unconjugated compound, provides an additional tool for testing the above conclusion. Since hepatic uptake and biliary excretion of diBSP and BSP are probably mediated by the same transport systems, the major difference in the movement of these compounds from blood to bile is that diBSP does not undergo intrahepatic conjugation whereas BSP does. If impaired conjugation of BSP was responsible for the decrease in BSP excretion in rats fed a protein-free diet for 2 days, one should not expect such a decrease in excretion of diBSP in similar experiments.

Materials and Methods. Male Sprague-Dawley rats, 200–300 gm were used in these studies. Special rat diets, purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, included a normal protein test diet, a protein-free diet and a protein-free diet containing 1% cysteine. All diets were supplemented with a vitamin fortification mixture. Phenol 3,6-dibromphthalein disulfonate in powder form was a gift from Hynson, Westcott and Dunning, Baltimore, Md. ^{35}S-labeled sulfobromophthalein sodium ($BS^{35}P$) was purchased from Volk Radiochemical Co., Chicago, Ill. Appropriate quantities of diBSP and $BS^{35}P$ were diluted with sterile saline (0.9% NaCl) for intravenous injections.

In the studies concerned with the effects of diet on diBSP excretion, groups of rats were initially fed the normal protein test diet for at least 3 days. After weighing, rats were either maintained on the same control diet, or fed the protein-free, or protein-free supplemented with 1% cysteine diet for an additional 2 days. Animals were weighed again, then under ether anesthesia their common bile ducts were cannulated with PE 10 tubing and the abdominal incision closed with metal clips. Ten mg of diBSP per 100 gm of body weight was administered into the femoral vein over a period of approximately 30 sec, and bile was collected for the ensuing 45 min in a tared bottle. At the termination of bile collection, blood was withdrawn from the aorta and the liver was removed and weighed.

Another group of studies was designed to examine whether diBSP and BSP competed with each other for transfer from blood to bile. Animals used in these experiments, maintained on a regular laboratory diet or control protein test diet, underwent bile duct cannulation as described above. Four rats were given $BS^{35}P$, 5 mg/100 gm of body weight; four additional rats received 5 mg $BS^{35}P$, plus 10 mg diBSP/100 gm of body weight simultaneously via intravenous injection; and six rats were injected with 15 mg diBSP/100 gm of body weight. Bile was collected for 45 min.

The animals remained under light ether anesthesia throughout the study in all experiments. Body temperature was controlled by keeping the rat covered from the neck down with a square of cloth and strapped to a heating pad which was adjusted to maintain a rectal temperature of 37–39°C (monitored at frequent intervals by a Tele-Thermometer obtained from Yellow Springs Instrument Co., Inc.). Unpublished observations in our laboratory, and the recent report of Roberts *et al.* (7), indicate that maximal biliary excretion of organic anions such as BSP and bilirubin in the rat is significantly affected by alterations in body temperature.

Four ml of 0.1 *N* KOH was added to 5 μl of bile containing diBSP and the optical density determined in a Beckman DU Spectrophotometer set at 570 mμ against a reagent blank consisting of 0.1 *N* KOH alone. The concentration of diBSP was calculated from a standard curve. The product of concentration in bile and bile volume (considered equivalent to the weight of bile collected) gives a value for total biliary excretion of diBSP. Plasma concentrations of diBSP were estimated by an adaption of the method of Gaebler used for BSP analysis (8). The BSP in bile was measured as described previously (5).

In the experiments in which both $BS^{35}P$ and diBSP were injected simultaneously, advantage was taken of the observation that equal concentrations (w/v) of these dyes yield approximately the same optical density at 570 mμ when the respective compounds are present in bile. Colorimetric analysis yielded a value for combined BSP and diBSP concentrations. The quantity of $BS^{35}P$ in each sample was calculated from the quantity of radioactivity determined to be present and the known specific activity (cpm/mg) of the injected BSP. Subtraction of the quantity of

TABLE I. diBSP Excretion into Bile in Rats on Control, Protein-free, and Protein-free with 1% Cysteine Diets.

Diet	No. of animals	Liver wt./ body wt. (%)	Plasma concentration at 45 min (mg/100 ml)	Bile volume (ml/100 gm per 45 min)	Bile diBSP concentration (mg/100 ml)	Quantity excreted (mg/100 gm per 45 min)
Control	9	3.5 ± 0.1[a]	19.6 ± 2.2	0.29 ± 0.02	1516 ± 66	4.45 ± 0.30
Protein-free	6	2.9 ± 0.1	26.6 ± 3.4	0.29 ± 0.02	1538 ± 83	4.40 ± 0.36
Protein-free 1% cysteine	5	3.8 ± 0.1	25.2 ± 2.1	0.31 ± 0.02	1543 ± 70	4.69 ± 0.34

[a] Values reported represent mean ± SE of the mean.

$BS^{35}P$ from the combined quantities of $BS^{35}P$ and diBSP yields a value for diBSP. For assay of radioactivity, 25 μl of bile was pipetted into a counting bottle containing 1.9 ml of absolute ethanol, the contents were mixed and 8 ml of phosphor (4 gm of 2,5-diphenyloxazole, 0.2 gm of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene) was then added. Samples were counted in a Beckman CPM-100 liquid scintillation system. After initial counting, internal standards were added to each vial to correct for any quenching.

Aliquots of bile were applied to Whatman no. 1 filter paper and subjected to descending chromatography as previously described (5) in a solvent system consisting of *n*-propyl alcohol, water, glacial acetic acid 10:5:1 v/v. The presence of diBSP compounds was ascertained by exposing the dried chromatograms to ammonia vapor. The possibility of conjugation of diBSP with glutathione was also examined by the *in vitro* enzymatic method described by Goldstein and Combes for assaying BSP–glutathione conjugation (9). In the present study, equimolar quantities of diBSP were substituted for BSP in the assay mixture.

Results. The volume of bile excreted in 45 min, the concentration of diBSP in bile, and the quantity of diBSP excreted in bile in 45 min were virtually the same in the rats on the control, protein-free, and protein-free supplemented with 1% cysteine diets (Table I). Plasma concentration of diBSP was somewhat higher in the protein-free and protein-free with 1% cysteine fed rats than in the control animals.

Chromatographs of bile collected after intravenous injection of diBSP revealed only one band with an *Rf* identical to that of the injected compound confirming the previous observations of Javitt (6). No evidence of conjugation of diBSP with glutathione was obtained with the assay method of Goldstein and Combes (9).

When $BS^{35}P$ was administered alone, an average of 4.16 mg/100 gm of body weight was excreted into bile in 45 min with an average concentration of 1179 mg/100 ml (Table II). The simultaneous injection of diBSP with $BS^{35}P$ resulted in decreases both in biliary excretion and in the concentration of each compound in bile. Composite excretion of both compounds, 8.86 μmoles/100 gm per 45 min when 20.71 μmoles were injected, was similar to the 8.28 μmoles of diBSP excreted when 22.13 μmoles of this single compound was administered.

Discussion. The present findings confirm the observations of Javitt (6) that phenol 3,6-dibromphthalein disulfonate is readily excreted into bile of the rat as the unconjugated compound. Javitt also found no evidence of diBSP conjugation with glutathione *in vitro,* and this was further supported by the use of another *in vitro* enzymatic technique (9) in the present study. Our studies, in addition, provide evidence that diBSP and BSP share common hepatic transport systems, since the simultaneous administration of both compounds resulted in decreased concentration and decreased excretion in bile of each compound, whereas composite excretion was apparently unaffected.

Of particular importance, were the findings that feeding rats a protein-free diet for 2 days

TABLE II. Biliary Excretion of $BS^{35}P$ and diBSP when Administered Singly or in Combination.

Compound injected	Dose (per 100 gm) (mg)	Dose (per 100 gm) (μmoles)	No. of animals	Concentration in bile (mg/100 ml) BSP	Concentration in bile (mg/100 ml) diBSP	Quantity excreted in bile (per 100 gm/45 min) BSP (mg)	Quantity excreted in bile (per 100 gm/45 min) BSP (μmoles)	Quantity excreted in bile (per 100 gm/45 min) diBSP (mg)	Quantity excreted in bile (per 100 gm/45 min) diBSP (μmoles)
$BS^{35}P$	5	5.96	4	1179 ± 60[a]		4.16 ± 0.18	4.96 ± 0.21		
$BS^{35}P$ + diBSP	5	5.96	4	879 ± 32[a]	872 ± 29[a]	3.30 ± 0.16[d]	3.94 ± 0.19	3.34 ± 0.31[e]	4.92 ± 0.46 (composite = 8.86 μmoles)
	10	14.75						4.45 ± 0.30	6.56 ± 0.44
diBSP	10	14.75	9		1516 ± 66				
diBSP	15	22.13	6		1516 ± 50			5.62 ± 0.68	8.28 ± 1.00

[a] Values reported represent mean ± SE of the mean.
[b–e] Significance of difference from respective single compound injection, *t* test, [b] $p < 0.01$; [c] $p = 0.001$; [d] $p < 0.01$; [e] $p = 0.05$.

exerted no significant effect on biliary excretion of diBSP. Since the same dietary regimen results in diminished excretion of BSP, accounted for by decreased excretion of conjugated BSP (5), the present studies provide strong supporting evidence for the conclusions that impaired conjugation accounted for decreased BSP excretion in the latter studies, and that conjugation of BSP is an important determinant of the maximal rate at which BSP is delivered from liver cells into bile.

Summary. Phenol 3,6-dibromphthalein disulfonate (diBSP) was shown to be excreted rapidly in the bile of rats as the unconjugated compound, confirming earlier observations of Javitt (6). In addition, it was demonstrated that diBSP and BSP share common hepatic transport systems. Finally, feeding a protein-free diet for 2 days did not affect the biliary excretion of diBSP, whereas the same diet was previously shown to result in decreased BSP excretion into bile accounted for by diminished excretion of conjugated BSP. The present findings support earlier conclusions that conjugation of BSP is an important determinant of the maximal rate of biliary excretion of BSP.

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