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Comparative Uptake of Sulfobromophthalein by Isolated Kupffer and Parenchymal Cells[1] (38827)

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Mammalian liver is composed of five distinct types of cells (1). Hepatocytes or parenchymal cells comprise 90–95% of the hepatic cellular weight and about 60–65% of the cell population. Reticuloendothelial (littoral, von Kupffer) cells constitute 5–10% of the liver weight and approximately 35% of the cellular population. Bile duct, connective tissue, and blood vessel endothelial cells constitute a small percentage of total cells and a minor component of liver mass.

Because of the previous inability to isolate Kupffer and parenchymal cells from the liver, the selective contributions of each major cell type to liver function have not been elucidated and parenchymal cells have been assumed to be largely responsible for many of the organ-specific functions of liver (2). However, the recent development of techniques for the isolation of cytologically homogeneous, structurally intact, metabolically active cells has provided an opportunity for studying the chemical, enzymatic, functional, and morphological characteristics of the two major cell types in the mammalian liver.

In a previous evaluation of the comparative biochemical characterization of Kupffer and parenchymal cells (3), distinct biochemical and functional differences have been demonstrated to exist between Kupffer and parenchymal cells (3, 4). Among the classical liver-function tests generally ascribed to parenchymal cells is the uptake of sulfobromophthalein (BSP). The BSP-clearance test, one of the classic clinical liver-function tests first described by Rosenthal and White (5), was originally suggested to be a Kupffer cell function (6, 7). Krebs and Brauer (8), using autoradiographic techniques, subsequently reported that BSP uptake was an event associated primarily with hepatic parenchymal cells. In contrast to these findings, Tovey (9) in 1967 stressed the participation of the Kupffer cells in the hepatic uptake of BSP.

To further elucidate the specific hepatic cell types involved in the uptake and conjugation of BSP, *in vitro* studies were undertaken using enzymatically isolated rat hepatic Kupffer and parenchymal cells. Brauer and Pessotti (10) previously demonstrated that BSP uptake by liver slices and the perfused liver was inhibited by addition of albumin or serum to the medium. The influence of serum or albumin on the uptake of BSP was evaluated to establish the employment of isolated hepatocytes in studies of BSP metabolism. Also, since hepatic dysfunction, as indicated by the altered plasma clearances of BSP, is a common manifestation of alcoholic liver disease (11), the effect of varying concentrations of ethanol on BSP uptake by isolated hepatic parenchymal cells was evaluated concomitantly.

Materials and Methods. Cell isolation. Hepatic parenchymal cells were isolated from male Sprague–Dawley rats (200–300 g), using Ontko's (12) modification of the original isolation procedure of Berry and Friend (13). The rats were anesthetized with ether and 300 units of sodium heparin were administered via an exposed inferior vena cava. The portal vein was cannulated with polyethylene tubing and the liver was perfused *in situ* for approximately 5 min with Ca^{2+}-free sodium bicarbonate-buffered Hanks' Balanced Salt Solution (HBSS) maintained at 37°C. The inferior vena cava was cut rostral to the liver to allow efflux of the perfusate. A mixture of 95% O_2–5% CO_2 was bubbled continuously through the perfusate to maintain proper pH (7.4). After perfusion the liver was removed and perfused with a 0.05% collagenase and 0.10% hyaluronidase solution in Ca^{2+}-free sodium

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bicarbonate-buffered Hanks' BSS maintained at 37°C. The enzyme solution was recirculated for 15 min after which the liver was gently disrupted and filtered first through cotton gauze and then through 10×× silk cloth. The resulting parenchymal cell suspension was washed and centrifuged at 50*g* for 1 min and washed twice with Hanks' incubation media (HCO_3^--free with Ca^{2+} buffered with 10 m*M* sodium phosphate [pH 7.4]). Kupffer cells were isolated using a modification of the method described by Lentz and Di Luzio (3), in which 1500 units of DNAse I (bovine pancreas, Sigma) were substituted for trypsin during the 15-min (37°C) incubation period. Cell counts were made with a hemacytometer and cell viability ascertained by trypan blue exclusion (14).

Cell incubation and BSP uptake procedures. Isolated hepatic parenchymal or Kupffer cells were suspended in bicarbonate-free Hanks' BSS containing 10 m*M* sodium phosphate buffer (pH 7.4). Cells were incubated with various concentrations (5, 10, 20%) of isologous rat serum or albumin (2.5, 5, or 10%). A 10-μg aliquot of BSP was added to each incubation flask in a total incubation volume of 8 ml.

In studies in which isolated cells were exposed to various concentrations of ethanol (100, 200, 400 mg/100 ml), the cells were preincubated for 30 min with ethanol prior to the addition of BSP.

Each flask was gassed with 95% O_2 and 5% CO_2, stoppered, and incubated at 37°C on a Dubnoff metabolic shaker.

BSP uptake by isolated cells was evaluated by removing appropriate aliquots from the incubation flasks at varying time periods. After centrifugation NaOH was added to the supernatant and the optical density of the sample determined at 580 nm (15). All experiments were carried out in triplicate with appropriate controls and standards. Since the BSP content of the medium did not change in the absence of cells, nor in the presence of heat-killed cells, a decrease in the BSP content of the medium reflected cellular uptake of BSP. Results are, therefore, expressed as net uptake (μg) of BSP per 10^7 cells. In all experiments Kupffer or parenchymal cell viability, as evaluated by trypan blue dye exclusion, did not vary >5% from the beginning to the end of any incubation period. Statistical significance was evaluated by the unpaired Student *t* test with a 95% confidence level.

Chromatographic studies. The method used for chromatographing BSP and the glutathione conjugate of BSP, i.e., BSP–GSH, was essentially that of Whelan and Plaa (16) employing cellulose powder, thin-layer chromatography plates. The solvent system was a *n*-butanol: glacial acetic acid: water (4:1:5) mixture. BSP was visualized by exposing the dried plates to a saturated NH_4OH atmosphere. The plates were also sprayed with ninhydrin (0.25%) in ethanol and heated at 37°C for 5 min to visualize the BSP–glutathione conjugate. BSP–GSH standard was prepared as described by Klaassen and Plaa (17). R_f values were determined for each BSP and BSP–glutathione conjugate spot.

Results. The enzymatic isolation of parenchymal cells resulted in an approximate yield of 2 × 10^8 cells (range 0.9–2.5) per liver which is in essential agreement with results of other investigators (18–20). The percentages of viable parenchymal cells as ascertained by trypan blue exclusion were 78–85%. Erythrocyte contamination of parenchymal cells was minimal, although some mononuclear cells were observed. Most of the isolated parenchymal cells displayed structural integrity; however, some (<2%) showed bleb formation on portions of their membranes.

The enzymatic isolation of Kupffer cells yielded approximately 6 × 10^7 cells (range 1.8–8.6) per liver. A similar yield using an enzymatic isolation procedure has been reported by Lentz and Di Luzio (3). Viability, determined by trypan blue exclusion, was approximately 90% in all Kupffer cell preparations. Differential counts of Kupffer cell preparations revealed an approximate 10% presence of lymphocyte-like cells.

The net uptake of BSP by isolated parenchymal cells was significantly greater than the uptake manifested by the isolated Kupffer cells (Fig. 1). The mean uptake and standard error in the latter cell population in μg/10^7 cells was 2.6 ± 1.7, 2.1 ± 0.1, and 2.5 ± 0.2 after 15, 30, and 60 min of incubation, respectively. The differential uptake of BSP by parenchymal cells was observed at all time

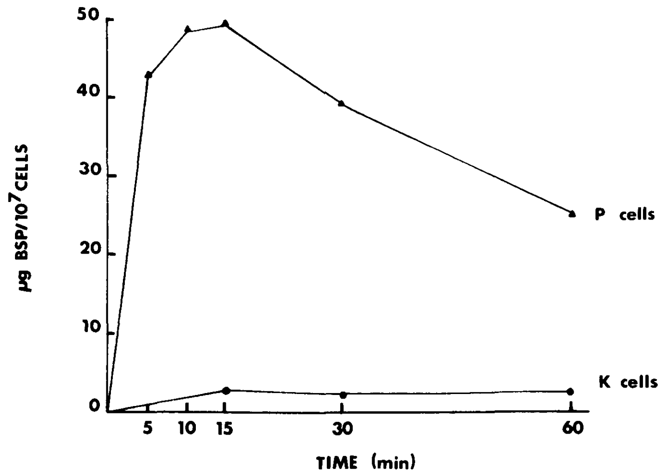

FIG. 1. Comparative uptake of BSP by isolated rat parenchymal and Kupffer cells incubated in buffer medium. Each point represents the mean of four experiments conducted in triplicate.

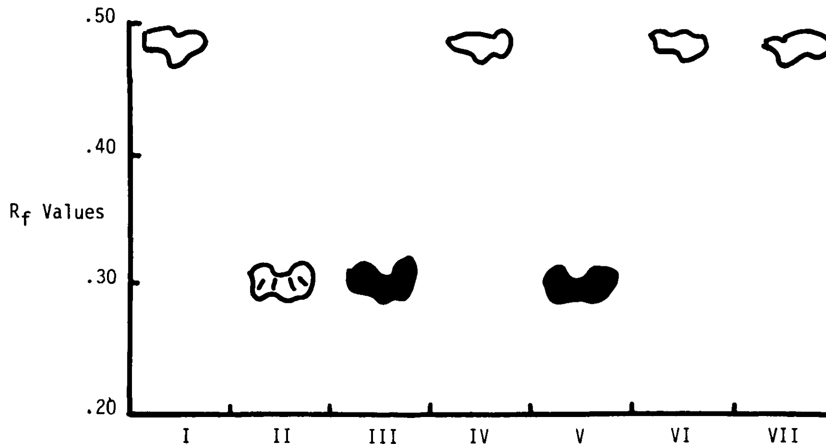

FIG. 2. Thin-layer chromatography of the incubation medium of isolated hepatic parenchymal and Kupffer cells incubated with BSP. System designations: I—BSP standard; II—glutathione standard; III—BSP-glutathione conjugate standard; IV—parenchymal cells incubated for 0 min with BSP; V—parenchymal cells incubated for 60 min with BSP; VI—Kupffer cells incubated for 0 min with BSP; VII—Kupffer cells incubated for 60 min with BSP. In contrast to the findings with parenchymal cells, BSP is not removed from the medium by Kupffer cells, nor is a BSP conjugate formed.

periods. Maximum uptake of BSP by the parenchymal cells of 49.8 ± 8.6 $\mu g/10^7$ cells was observed at 15 min of incubation. A decrease in BSP content of the parenchymal cells occurred after that period as reflected by an elevation in the concentration of BSP in the incubation medium. Thin-layer chromatographic studies revealed the absence of free BSP and the appearance of conjugated BSP in the medium after 60 min of incubation (Fig. 2). These events were specifically associated with parenchymal cells, as the absence of free BSP from the medium and the formation of BSP–GSH did not occur with Kupffer cells (Fig. 2). The R_f value for the BSP standard was comparable to the R_f values of the BSP present in the incubation medium of the parenchymal cell system at zero time and the Kupffer cell medium at both zero time and 60 min. Similarly, the R_f values of the BSP–GSH standard and that of samples obtained from the medium of parenchymal incubation mixture at 60 min were comparable.

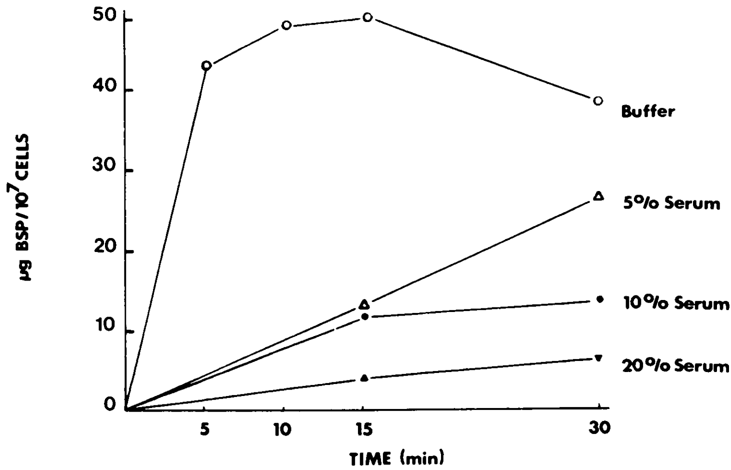

FIG. 3. Influence of varying concentrations of serum on the net uptake of BPS by isolated parenchymal cells. Values represent the mean of four experiments conducted in triplicate.

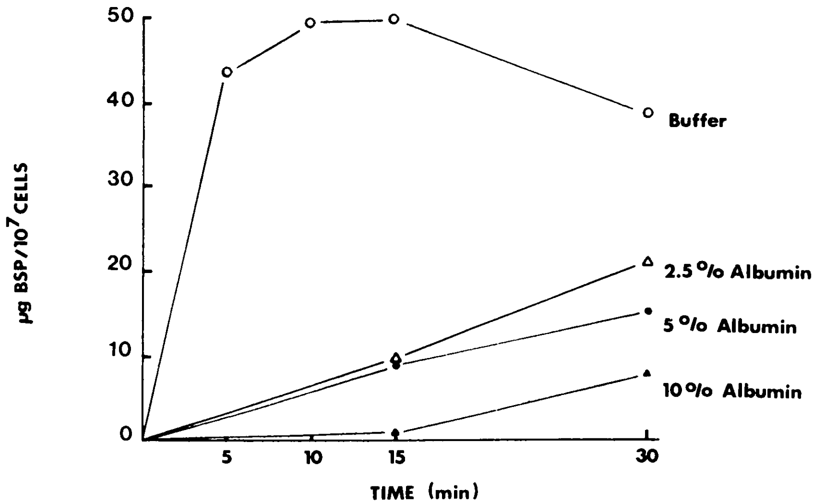

FIG. 4. Influence of varying concentrations of albumin on the net uptake of BSP by isolated parenchymal cells. Bovine serum albumin (Sigma Type V) was used. All values represent the mean of four experiments conducted in triplicate.

In two additional studies employing various concentrations of heat-killed parenchymal cells (10 min at 56°C), no loss of the BSP from the medium was manifested, indicating that viable parenchymal cells were needed for BSP uptake. The passive adsorption of BSP on the membrane of the parenchymal cell is, therefore, not a contributing factor in the uptake of BSP. In control studies in which cells were not employed in the system, the concentration of BSP in the incubation medium remained unaltered.

The rate and maximum uptake of BSP were inversely related to the concentration of serum (Fig. 3) or albumin (Fig. 4) in the incubation medium. With a 10% serum concentration, the uptake was reduced from 38.9 ± 7.7 $\mu g/10^7$ cells to 15.9 ± 3.4 at the 30-min period. Increasing the albumin concentration to 2.5 and 10.0% also profoundly inhibited BSP uptake at all time periods studied. The mean values and standard errors in the nonalbumin medium at 15 and 30 min of incubation were 29.8 ± 12.4

TABLE I. FAILURE OF ETHANOL TO MODIFY SULFOBROMOPHTHALEIN UPTAKE BY ISOLATED HEPATIC PARENCHYMAL CELLS[a]

Ethanol (mg/100 ml)	Net uptake of BSP, $\mu g/10^7$ cells	
	15 Min	30 Min
—	23 ± 3	29 ± 2
100	22 ± 4	27 ± 3
200	23 ± 4	27 ± 1
400	22 ± 8	28 ± 4

[a] Parenchymal cells were incubated with saline or varying concentrations of ethanol for 30 min prior to addition of BSP. Incubation media contained 10% serum. Values are the mean and standard error of three experiments each conducted in duplicate.

and 38.9 ± 7.7, respectively. In contrast, with 2.5% albumin in the medium, the values were 9.9 ± 5.1, 21.6 ± 2.9 at 15- and 30-min periods, respectively. A significantly greater inhibition of BSP uptake with 10% albumin was observed at the 15- and 30-min period when BSP uptake, 0.8 ± 0.4 and 8.5 ± 3.5 $\mu g/10^7$ cells was observed. Additional experiments revealed that the inability of Kupffer cells to take up BSP from the medium was not altered by variations in cell number or serum concentration.

Incubation of isolated parenchymal cells with either 100 mg/100 ml, 200 mg/100 ml, or 400 mg/100 ml of ethanol did not alter BSP uptake (Table I). At the concentrations of ethanol employed, no direct cytotoxicity of ethanol could be demonstrated on the isolated hepatocytes.

Discussion. The BSP test is a sensitive index for the evaluation of hepatic function and the identification of subclinical abnormalities of liver dysfunction (21–25). It has been suggested that upon the administration of BSP an almost instantaneous binding of the BSP with plasma proteins occurs (25). Analysis of sera containing BSP has revealed that approximately one-third of the BSP is bound to globulins, predominantly alpha-1-lipoprotein, and two-thirds is bound to albumin (26).

In initial attempts to delineate the principal hepatic cell population, i.e., Kupffer or parenchymal cell, involved in the uptake of BSP, reticuloendothelial (RE) blockade induced by India ink was employed as a means of assessing the contribution of the Kupffer cell to BSP metabolism. Mills and Dragstedt (6) and Wirts and Cantarow (7) reported the retention of BSP after the intravenous administration of India ink to dogs. These investigators postulated that the defect was the result of an impairment in the uptake of BSP by the RE cells due to colloidal blockade. Based upon these observations, they concluded that Kupffer cells were responsible for the clearance of BSP from the plasma. However, Shore and Zilversmit (27) subsequently demonstrated that India ink promotes an hepatotoxic reaction which was not due to the colloidal carbon component of India ink and thus questioned the entire procedure of RE blockade. Krebs and Brauer (8) demonstrated by means of autoradiography that BSP uptake appeared to be a function of the hepatic parenchymal cells. The present study using enzymatically isolated rat hepatic Kupffer and parenchymal cells confirms the original observation by Krebs and Brauer (8) that hepatic uptake of BSP is an exclusive function of the parenchymal cell population and negates the concept of Tovey (9) that Kupffer cells functionally participate in BSP removal.

Among factors which were found to significantly influence BSP uptake by parenchymal cells was the presence of either serum or albumin in the incubation medium. These findings agree with the earlier work by Brauer and Pessotti which demonstrated that BSP uptake by liver slices as well as perfused livers was significantly diminished by the addition of albumin or serum to the incubation medium (10). The inhibitory effect of albumin on BSP uptake was dose dependent and related to formation of protein–BSP complexes (10). This inhibitory effect may be the result of competition between the protein in the medium and the parenchymal cell for BSP (10, 25) rather than the result of a nonspecific inhibitory effect of serum on the isolated parenchymal cells as previously proposed by Garvey (28).

The finding that BSP uptake is inversely related to the concentration of albumin suggests that the extent of hepatic injury in in-

dividuals with chronic liver disease associated with hypoalbuminemia might be underestimated when BSP is employed. While Brauer and Pessotti (10) observed the decrease in BSP uptake by isolated liver slices, as well as perfused livers by addition of albumin to the system, the possible implications of decreased albumin levels to enhancement of BSP removal rates in various clinical situations of chronic liver injury are as yet to be defined.

Associated with the parenchymal cell uptake of BSP was the subsequent formation of a BSP–GSH conjugate. The conversion of BSP to the glutathione conjugate appears to be a principal mechanism in the metabolism and excretion of the dye by mammals (29). The present study has demonstrated that hepatic BSP–glutathione-conjugating activity appears to reside only in the parenchymal cell and not the Kupffer cell as a BSP–GSH conjugate was never observed in the absence of parenchymal cells in the incubation medium.

Since chronic alcoholism has been demonstrated to significantly depress plasma clearance of BSP (11) and since BSP uptake was demonstrated to be a function of hepatic parenchymal cells, the effect of ethanol on BSP uptake by isolated hepatic parenchymal cells was evaluated. Ethanol at concentrations of 100–400 mg/100 ml in the incubation medium did not cause any alteration in BSP uptake by the isolated parenchymal cells. Concentrations of ethanol used in the present study had previously been demonstrated by Ontko (30) to increase fatty acid esterification and triglyceride concentration in isolated parenchymal cells. The inability to detect a direct effect of ethanol on hepatic uptake of BSP was also observed by Kotelanski *et al.* (31) using an isolated perfused rat liver and concentrations of ethanol previously demonstrated by Williamson *et al.* (32) to produce diverse dose-related alterations in intermediary metabolism in the perfused rat liver. These composite findings suggest that the altered BSP clearance observed in chronic alcoholism may be attributable to hepatic injury and not to a direct effect of ethanol on BSP uptake.

The present studies clearly establish the parenchymal cell as the specific cell type involved in the uptake of BSP. Further, these experiments illustrate the potential use of isolated cells to provide a model system for the rapid and direct assessment of hepatotoxic agents on hepatic cell populations and functions.

Summary. The relative role of specific liver cells in the uptake of sulfobromophthalein (BSP) was ascertained by utilizing enzymatically isolated rat hepatic Kupffer and parenchymal cells. Kupffer cells demonstrated the ability neither to remove BSP from the incubation medium nor to form a BSP–glutathione conjugate. In contrast, parenchymal cells removed BSP from the medium and formed a BSP–glutathione conjugate. The rate and maximum uptake of BSP by the parenchymal cells were inversely related to the concentration of serum or albumin in the incubation medium. In an effort to evaluate the influence of ethanol on BSP uptake, parenchymal cells were incubated in the presence of varying concentrations of ethanol. No alteration in BSP uptake was induced by the prior addition of ethanol to the incubation medium. The uptake and conjugation of BSP are exclusive functional expressions of the hepatic parenchymal cell population.

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