BROMSULFOPHTHALEIN UPTAKE BY ISOLATED LIVER PARENCHYMAL CELLS

C.F.A. van Bezooijen, T. Grell and D.L. Knook

Institute for Experimental Gerontology TNO,
151 Lange Kleiweg, Rijswijk, The Netherlands

Received January 26, 1976

SUMMARY: The specific liver function of removing foreign compounds from the serum was investigated by measuring the uptake of |^{35}S|bromsulfophthalein by isolated liver parenchymal cells. To obtain a maximum uptake, the parenchymal cells in cell concentrations ranging between 0.05 and 0.4 x 10^6 cells/ml were incubated with a dose of 30 nmoles |^{35}S|bromsulfophthalein/ml for 15 min at 37°C. An uptake of 2.87 ± 0.18 nmoles bromsulfopthalein/10^6 cells was measured. The saturation of the rate of bromsulfophthalein uptake with increasing amounts of bromsulfophthalein in the medium, the ability to take up free bromsulfopthalein against a concentration gradient and the dependence of the uptake mechanism on temperature and metabolic energy suggest the presence of an active carrier system for the uptake of bromsulfophthalein by liver parenchymal cells.

INTRODUCTION: One of the major functions of the liver is the transfer of organic anions such as bilirubin, several steroids and drugs from the plasma into the parenchymal cells. The capacity of the liver to take up organic anions can be determined *in vivo* by the clinical bromsulfophthalein (BSP) retention test. In this test, intravenously injected BSP combines mainly with the serum albumin and this complex is transported to the parenchymal liver cells (1). After dissociation from albumin, BSP is transferred from the blood into the parenchymal cells. In the cytoplasm of the cells, the BSP is partially bound by Y protein (ligandin) (2). The bound BSP is then conjugated with glutathione by glutathione S-transferase B, which may be identical to ligandin (3). Both the unconjugated and the conjugated BSP are finally excreted into the bile canaliculi.

Homogeneous liver parenchymal cell suspensions are uniquely suitable for studying liver-specific functions and for quantifying these functions on a cellular basis. A method was developed to quantitate one of these specific liver functions, *viz.* the uptake of BSP, with hepatocyte suspensions. The nature of the processes by which BSP and other organic anions are transported across the cell membrane will also be described in this report.

Abbreviation: BSP, bromsulfophthalein.

MATERIALS AND METHODS: Three-month-old female WAG/Rij rats, weighing 130-160 g, were used. The maintenance conditions have been previously described (4). The standard medium employed in all experiments had the same composition as described earlier (4) except that the fructose, glucose and sucrose concentrations were 20, 20 and 150 mM, respectively. Parenchymal cells were isolated from rat liver as reported earlier (4) with the following modifications: A preperfusion was performed with standard medium at a rate of 15 ml/min for 15 min. After the preperfusion, the liver was perfused by a recirculation technique with standard medium containing collagenase (0.05%) and hyaluronidase (0.10%) at a rate of 15 ml/min for 20 min. The oxygen tension in the medium was maintained at at least 50×10^3 Pa. These modifications resulted in a higher yield of viable cells, which ranged from $30\text{-}40 \times 10^6$ cells/g wet weight of liver.

Trypan blue uptake, the volume of the isolated cells and cell concentrations were determined as previously described (4). Rat serum albumin was measured according to De Leeuw-Israel et al. (5).

To determine the amount of BSP uptake, a known number of cells in 1.5 ml of standard medium was incubated with $|^{35}S|$BSP at 37°C under an atmosphere of 95% O_2 and 5% CO_2 with constant shaking (100 oscillations/min). Immediately after the addition of $|^{35}S|$BSP to the cell suspension and after various incubation periods, 0.5 ml samples were withdrawn and added to 5 ml ice-cold 0.3 M phosphate buffered saline (pH 7.4). The cells were washed three times with 5 ml ice-cold phosphate buffer with interim centrifugation at 100 x g. The cell pellet was then dissolved in 1 ml soluene and the radioactivity of the samples was determined. Values obtained after incubation were corrected for the non-specific binding of BSP on the cellular membrane represented by the amount of radioactivity found in the sample withdrawn immediately after the addition of $|^{35}S|$BSP to the cell suspension.

The amount of BSP bound to cytoplasmic proteins in the parenchymal cells was determined as follows. The first steps of the procedure were identical to the method described above for measuring the total amount of BSP uptake by parenchymal cells. After washing the cell pellet was resuspended in 5 ml ice-cold 10% trichloroacetic acid and stored for at least 30 min at 0°C. The resulting precipitate was washed two times with 5 ml ice-cold 5% trichloroacetic acid. The final pellet was solubilized with 1 ml soluene and the radioactivity measured. Separation of free BSP and glutathione-BSP was performed essentially according to the method of Freundt (6) employing cellulose powder thin-layer chromatography.

RESULTS:

$|^{35}S|$BSP uptake by isolated parenchymal cells: To determine the time course of BSP uptake, isolated parenchymal cells (290,000 cells/ml) were incubated with 30 nmoles $|^{35}S|$BSP/ml incubation medium and the amount of BSP taken up was determined after various time intervals. The uptake appeared to be linear for the first 10 min and reached a plateau at about 12 min. Thereafter, the BSP uptake did not change up to 45 min of incubation. Fig. 1 shows the correlation between the concentration of $|^{35}S|$BSP in the incubation medium and the amount of BSP taken up in 15 min by the isolated cells. For cell suspensions consisting of viable parenchymal cells maximum BSP uptake takes place at about 25 nmoles BSP/ml incubation medium.

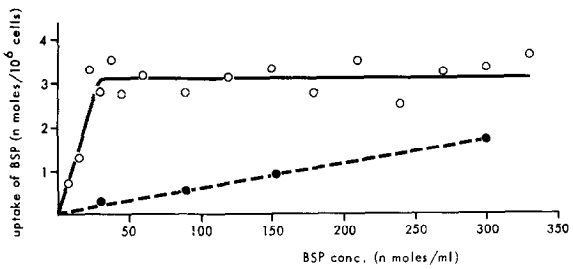

Fig. 1. The uptake of $|^{35}S|$BSP by a suspension of viable (O——O) and non-viable (●– – –●) parenchymal cells as a function of the concentration of BSP in the incubation medium. Sixteen cell suspensions of viable cells with a cell concentration of 120,000 cells/ml and four cell suspensions of nonviable cells with a cell concentration of 300,000 cells/ml were incubated at 37°C for 15 min with various amounts of BSP in the medium.

On the basis of the results mentioned above, the BSP uptake in subsequent experiments was measured with 30 nmoles $|^{35}S|$BSP/ml medium after 15 min of incubation. These test conditions guarantee that small changes in the BSP uptake by the cells due to internal or external influences can be easily detected.

The plateau level in Fig. 1 was observed only for suspensions consisting almost entirely of viable cells. Cell suspensions with a high number of non-viable (trypan blue stained) cells did not show a plateau level but the amount of BSP taken up by the cells showed a linear increase with increasing concentrations of BSP in the incubation medium.

The amount of BSP taken up by the isolated hepatocytes was proportional to the number of cells present in the incubation medium up to a concentration of 0.4×10^6 cells/ml medium. In subsequent experiments, therefore, cell suspensions ranging from 0.05×10^6 to 0.4×10^6 cells/ml were used.

With due observance of the experimental conditions defined above, viable liver parenchymal cells from 3-month-old rats appeared to take up 2.87 ± 0.18 nmoles BSP/10^6 cells. Expressed on a protein basis, 1.19 ± 0.07 nmoles BSP/mg protein were taken up by the isolated hepatocytes. This latter calculation was based on a content of 2.41 mg protein per 10^6 cells (4).

In vivo, a substantial fraction of BSP is carried to the parenchymal cells by plasma albumin. The physiological concentration of albumin in the serum of 3-month-old WAG/Rij rats was found to be 4.6%. Addition of 5% albumin to the incubation medium inhibited the uptake of BSP by the isolated cells, as demonstrated in Table 1.

Table 1. Effect of bovine serum albumin on the amount of BSP taken up by isolated liver parenchymal cells

addition	n	Uptake of BSP (nmoles/10^6 cells) mean	S.E.	P
albumin	3	0.07	0.02	<0.0001
none	2	2.70	0.16	

The parenchymal cell suspensions were incubated at 37°C for 15 min with 30 nmoles BSP/ml.

Nature of the BSP transport across the hepatocyte membrane: In order to discriminate between a passive diffusion process or a carrier transport mechanism for the BSP uptake, the rate of BSP uptake by the cells was measured. The cells were incubated with various amounts of BSP for 5 min, a period during which the uptake of BSP is linear with time. Fig. 2 shows that, with increasing amounts of BSP in the incubation medium, a plateau level is reached for the rate of uptake by the cells. The saturation of the rate of uptake at higher BSP concentrations indicates that the transport of BSP across the cell membrane is of the carrier type.

For a discrimination between the two possible types of carrier transport, viz. facilitated diffusion and active transport, it is necessary to determine whether or not the transport of BSP is operating against a concentration gradient. For this purpose, the amount of free BSP that accumulated in the isolated

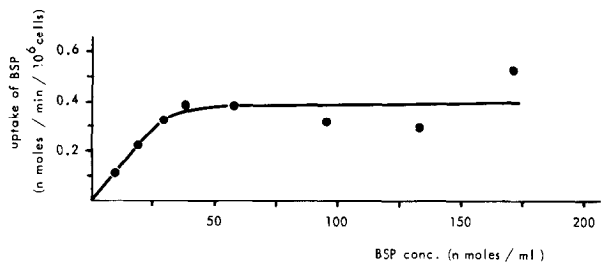

Fig. 2. The relationship between the rate of BSP uptake by the cells and the concentration of BSP in the incubation medium. Data represent the mean of 3 experiments. Eight cell suspensions were incubated at 37°C for 5 min with different amounts of BSP in the medium.

cells had to be determined. The amount of BSP bound by ligandin appeared to be 1.06±0.34 nmoles BSP/10^6 cells. The total amount of BSP taken up by the cells amounted to 2.87±0.18, as described above. From these results, it could be calculated that about 40% of the total amount of BSP taken up by the cells was bound by ligandin. Thin layer chromatography revealed that, of the amount of BSP in the parenchymal cells not bound by ligandin, 95% and 5% could be considered as free BSP and conjugated BSP, respectively. It was mentioned above that the amount of BSP taken up by the cells was 2.87 nmoles BSP/10^6 cells. From this value and from a measured mean volume of 5405 μ^3 for the parenchymal cells isolated from 3-month-old WAG/Rij rats, the concentration of free BSP within a cell could be calculated to be about 310 nmoles BSP/ml parenchymal cell volume. In view of the impermeability of various cell organelles for BSP, the actual concentration of free BSP in the cytoplasm of the hepatocytes will be even higher than the mentioned value. Thus the concentration of free BSP within the cells is at least 10 times higher than the concentration of free BSP in the incubation medium (30 nmoles BSP/ml). This suggests active transport of BSP into the cell.

The dependency on temperature is another characteristic of active transport. It was found that the amount of BSP taken up was significantly higher at 37°C than at lower temperatures (Fig. 3). A ratio (Q10) of 2.2 for the amount of BSP taken up by the parenchymal cells at 37 and 27°C, respectively, could be calculated. This value correlates well with ratios of 2-3 measured for active transport of amino acids into tissues (7).

To test whether the uptake of BSP is dependent on cellular energy, the rate of uptake was measured in the presence of DNP or KCN (Table 2). KCN and DNP significantly reduced the rate of BSP uptake. In separate experiments, the concentrations of KCN and DNP employed caused a marked inhibition or uncoupling of the endogenous respiration of the cells.

DISCUSSION: The important liver function of removing foreign substances from the serum can be examined by means of determining the uptake of BSP. In preliminary reports, it was mentioned that, after isolation, the single parenchymal cells retained the capacity to take up BSP and that the addition of bovine serum albumin to the incubation medium inhibited the uptake of BSP (8, 9). This finding has recently been confirmed by Stege *et al.* (10).

The mechanism by which BSP or other organic anions are transferred from the plasma into the liver parenchymal cells is still unknown. Arias (11) suggested that specific unidirectional anion carriers might not exist on the sinusoidal surface of the plasma membrane and that organic anion transfer into the cell probably involved nonionic diffusion. The same suggestion was made by Von Bahr

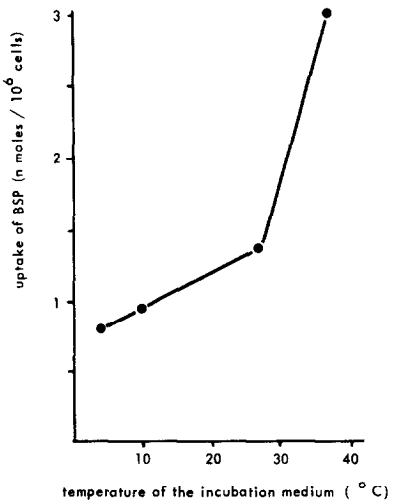

Fig. 3. Dependency of the amount of BSP uptake in 15 min by the isolated hepatocytes on the temperature of the incubation medium. Four cell suspensions were incubated for 15 min with 30 nmoles $|^{35}S|$BSP/ml at the indicated temperatures. The data plotted in the figure are the mean of five experiments.

Table 2. Inhibition effect of KCN and DNP on the rate of BSP uptake by isolated liver parenchymal cells

addition	final conc. (M)	n	Rate of BSP uptake (nmole/min/10^6 cells) mean	S.E.	% inhibition	P
KCN	$5x10^{-4}$	14	0.138	0.024	56	
						<0.01
none		11	0.316	0.064		
						<0.02
DNP	$5x10^{-4}$	11	0.141	0.017	55	

The various cell suspensions were incubated at 37°C for 5 min with 30 nmoles BSP/ml.

et al. (12) who measured the uptake mechanism of hexobarbital by isolated liver parenchymal cells. The results presented in this paper, which show that the rate of BSP uptake is not proportional to the BSP concentration in the medium but approaches a maximum level, are indicative for a carrier type of BSP transport into the viable liver parenchymal cell. No saturation of the rate of uptake was observed for cells stainable by trypan blue, suggesting a passive diffusion of

BSP into damaged cells. The observations that BSP was transferred across the cell membrane against a concentration gradient in viable cells and that the BSP uptake was dependent on temperature and energy strongly suggest that BSP is transported into the liver parenchymal cells by an active transport system.

The results obtained on the BSP uptake by isolated cells make it possible to compare in vitro data with the in vivo situation. Now the relative storage capacity in vivo can be compared with the relative storage capacity in vitro. The relative storage capacity in vivo is defined as the number of mg BSP taken up into storage/mg BSP in 100 ml of plasma/kg body weight. De Leeuw-Israel et al. (13) calculated an in vivo value of 1.16 mg BSP/mg BSP/100 ml/kg body weight. The relative storage capacity in vitro may be defined as the amount of BSP/mg BSP in 100 ml of incubation medium/kg body weight taken up by the total number of parenchymal cells/liver. An in vitro value of 4.35 mg BSP/mg BSP/100 ml/kg body weight could be calculated. This calculation was based on the measured liver weight of 6.0 g for 3-month-old rats and on the assumption that the parenchymal cell mass contains 114×10^6 cells/g liver wet weight (14). The obtained in vivo and in vitro values show that the capacity of the parenchymal cells to take up BSP is not negatively influenced by the isolation procedure. Preliminary results obtained by thin layer chromatography indicated the appearance of glutathione-conjugated BSP in the medium during the incubation of the cells. It may be concluded, therefore, that the isolated parenchymal cells are capable of performing all of the various steps of the BSP clearance mechanism.

ACKNOWLEDGEMENTS: The authors wish to thank Dr. K.J. van den Berg and Dr. C.F. Hollander for their interest, advice and critical reading of the manuscript. The skilled technical assistance of Mrs. G.J. Beyersbergen-Van Oosten, Mrs. A.J. van de Siepkamp-De Jong, Miss E.C. Sleyster, Miss F.G. Westerhuis, and Mr. N. Blansjaar is gratefully acknowledged.

REFERENCES:

1. Baker, K.J., and Bradley, S.E. (1966) J.Clin. Invest, 45, 281-287.
2. Mishkin, S., Stein, L., Gatmaitan, Z., and Arias, I.M. (1972) Biochem. Biophys. Res. Comm, 47, 997-1003.
3. Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M., and Jakoby, W.B. (1974) Proc. Nat. Acad. Sci. USA, 71, 3879-3882.
4. Van Bezooijen, C.F.A., van Noord, M.J., and Knook, D.L. (1974) Mech. Age. Dev, 3, 107-119.
5. De Leeuw-Israel, F.R., Arp-Neefjes, J.M., and Hollander, C.F. (1967) Exp. Geront. 2, 255-260.
6. Freundt, K.J. (1973) Z. Gastroenterologie, 11, 565-572.
7. Van den Berg, K.J. (1974) The role of amino acids in the mitogenic activation of lymphocytes, Thesis, University of Leiden.
8. Van Bezooijen, C.F.A., Van Noord, M.J., and Knook, D.L. (1973) Digestion, 8, 463-464.

9. Van Bezooijen, C.F.A., and Knook, D.L. (1974) Scand. J. Clin. Lab. Invest. 34, suppl. no. 141, 30.
10. Stege, T.E., Loose, L.D., and Di Luzio, N.R. (1975) Proc. Soc. Exp. Biol. Med, 149, 455-461.
11. Arias, I.M. (1972) Seminars in hematology, 9, 55-70.
12. Von Bahr, C., Vadi, H., Grundin, R., Moldeus, P., and Orrenius, S. (1974) Biochem. Biophys. Res. Comm, 59, 334-339.
13. De Leeuw-Israel, F.R., Hollander, C.F., and Arp-Neefjes, J.M. (1969) J. Geront, 24, 140-142.
14. Seglen, P.O. (1973) Exptl. Cell Res, 82, 391-398.