EVIDENCE FOR THE EXISTENCE OF A CARRIER FOR BROMOSULPHONPHTHALEIN IN THE LIVER CELL PLASMA MEMBRANE

M. FREZZA, C. TIRIBELLI, E. PANFILI* and G. SANDRI*

Istituto di Patologia Speciale Medica e Istituto di Chimica Biologica Universita di Trieste, Trieste, Italy*

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1. Introduction

It is well known that bilirubin passes from blood to bile by a selective process, which is common to other organic anions such as steroids, drugs, bromosulphonphthalein (BSP), Indocyanin Green (ICG), Rose Bengal and so on. This process moreover is very rapid: 5 min after the injection of radioactive bilirubin, 63% of the dose is found in the liver, of which 75% is recovered in the supernatant [1,2]. The same phenomenon occurs for BSP and ICG, but not for albumin [3]. Recently [4] the presence of intrahepatic proteins (Y and Z), which display high affinities for bilirubin and other organic anions, has been demonstrated. This finding has led Arias [5] to assume that the uptake of these substances was strictly correlated with the two proteins. He assesses more precisely that the process was dependent on 'the amount and binding affinity of albumin in plasma, and the amount and binding affinities of Y and Z in the cytoplasm. Influx is largely determined by dissociation of anion from protein in blood and nonionic diffusion across the plasma membrane of the liver cell' [5]. From data reported in literature [6,7] it may be calculated that the absolute amount of bilirubin inside the liver is approximately twice as much as that in the plasma. The concentration difference should be higher [7]. This is of course not the free bilirubin concentration. In fact, both in the plasma and in the liver the bulk of the bilirubin must be bound to albumin and to Y and Z respectively. It may be calculated from the total amount of albumin and the dissociation constant of the bilirubin–albumin complex that the concentration of free bilirubin in the plasma is lower than 10^{-8} M. No data however, are available concerning free bilirubin concentration inside the liver cell.

In order to investigate the mechanism of bilirubin uptake across the liver cell plasma membrane, we studied the binding affinity of BSP both to plasma proteins and liver cell sap. We decided to use BSP because it behaves very similar by to bilirubin with respect to blood clearance and excretion. We chose to use crude fractions (total serum and cytosol) rather than purified components to better simulate physiological conditions.

2. Materials and methods

Serum and liver from Wistar rats were used. Serum was prepared in the usual way and stored at −20°C until used. Livers were perfused in situ immediately after sacrifice with ice-cold saline solution through the hepatic veins, until all traces of blood were removed. All the following operations were performed at 0°C. The liver was then removed, weighed and suspended in 0.25 M sucrose containing 0.01M phosphate buffer, pH 7.4 according to Levi et al. [4]. Homogenization was carried out at 33% w/v with a motor-driven 50ml Potter homogenizer equipped with a Teflon pestle. This suspension was immediately spun down in a Spinco ultracentrifuge at 105 000 *g* for 120 min. The pellet was discarded, and the supernatant, after removal of the lipid layer, was kept at −20°C until used.

Protein contents of serum and liver supernatant were determined by biuret reaction according to Gornall et al. [8]. Bromosulphonphthalein sodium was obtained from DADE (USA). Gel filtration chromatography of the supernatant was carried out

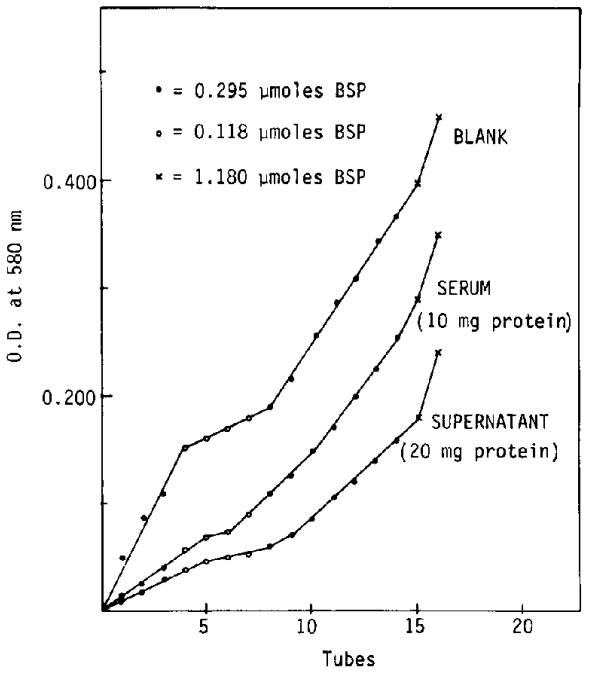

Fig. 1. BSP binding to proteins shown by flow dialysis. Flow rate was 60ml/hr, and time collection 12 min per tube. See text for details.

in a cold room using a Sephadex G-75 column, equilibrated in 0.01 M phosphate buffer, pH 7.4. About 3.5 ml of supernatant (100 mg protein), brought to room temperature, were added to 5.9 nM BSP and kept at 25°C for 5 min. This material was loaded on to the column and eluted with the above mentioned buffer. BSP binding capacity was tested by means of continuous flow dialysis, essentially according to Colowick and Womack [9]. The system consists of two superimposed chambers, separated by a Kalle dialysis membrane preincubated overnight in 0.295 μM BSP. Both chambers are stirred. The upper part of the cell contains 2 ml of 0.01 M phospahte buffer, pH 7.4, and the protein sample. Into this compartment increasing BSP additions were made, starting from 0.147 μM and reaching 2.094 μM. A continuous flow was maintained in the lower chamber (0.1 ml) by an LKB Perpex pump, using the above-mentioned buffer. The concentration of the anion in the effluent from the lower chamber was measured spectrophotometrically at 580 nm after alkalinization. Values obtained were proportional to the free anion in the upper cell. A suitable blank, devoid of protein, was run under the same conditions. Fig. 1 shows the curves obtained in a typical experiment, from which we can calculate, at each

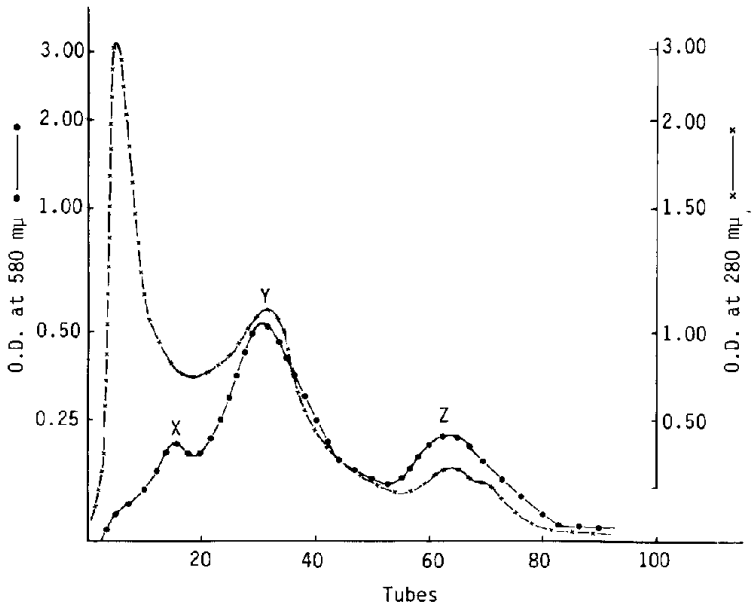

Fig. 2. Elution profile from Sephadex G-75 (3 cm x 46 cm) of rat-liver supernatant (60 mg protein). Elution with 0.01M phosphate buffer pH 7.4, at a flow rate of 360 drops/hr. Each tube contained 4.5 ml. Proteins were followed at 280 nm, and protein-linked BSP, after alkalinization, at 580 nm

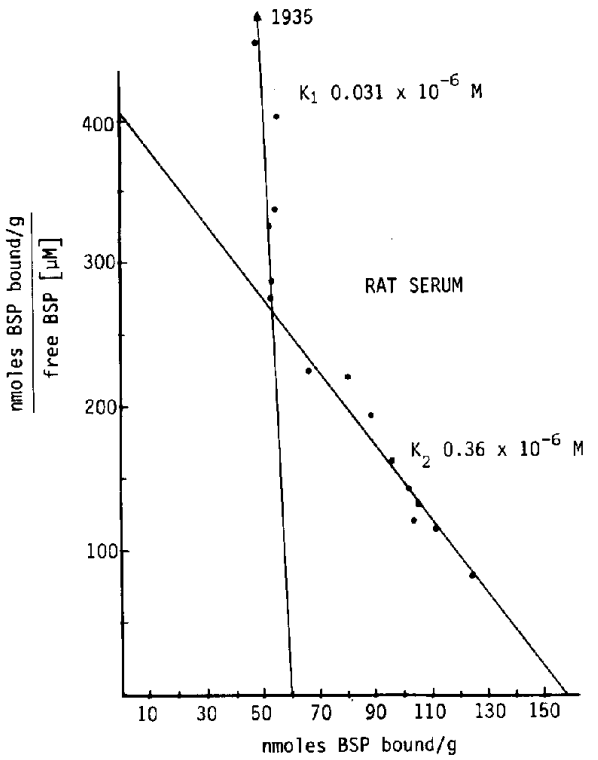

Fig. 3. Scatchard plot of BSP binding to rat-serum proteins. Protein content 10 mg in 0.01 M phosphate buffer, pH 7.4.

addition of BSP, the percentage of free BSP, its actual concentration and the BSP bound per mg of protein. These results were plotted by the graphical method of Scatchard [10]. The experimental values were fitted by least squares using a computer.

3. Results and discussion

The presence in the supernatant of the organic anions binding proteins Y and Z was demonstrated by the elution profile from Sephadex G-75, shown in fig. 2. This result correlates extremely well with the findings already presented by Levi et al. [4].

Fig. 3 is a Scatchard plot of BSP binding to rat serum. Clearly, two classes of affinity binding sites are present, one with high and the other with low affinity. The nmoles of BSP bound per mg of protein are 60 and 155 respectively. Dissociation constants derived from the graph are 0.031 X 10^{-6} and

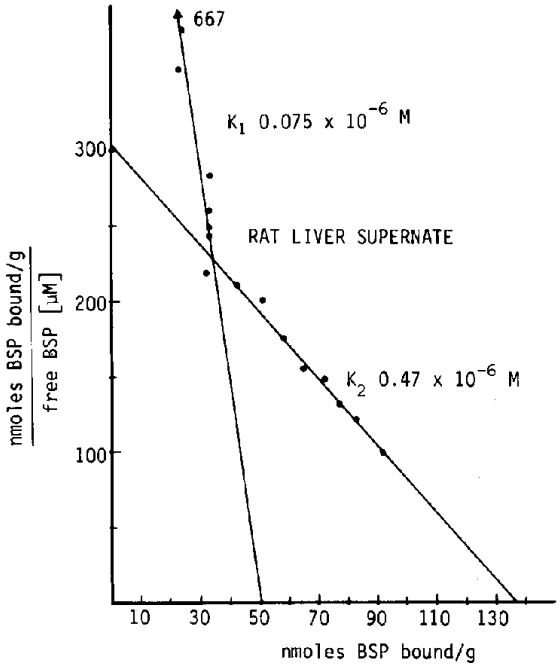

Fig. 4. Scatchard plot of BSP binding to rat-liver supernatant proteins. Protein content 20 mg in 0.01 M phosphate buffer, pH 7.4, containing 0.25 M sucrose.

0.363 X 10^{-6} M for the high and low affinity sites respectively. These data are in good agreement with data reported in literature for the affinity constants of rat serum albumin (K_1 = 1.7 X 10^7 and K_2 = 1.4 X 10^6) [5]. It may be calculated from these data that the binding capacity of serum may be entirely accounted for by its albumin content.

Fig. 4 shows the BSP binding capacity of rat-liver supernatant. In this case two classes of binding sites are also present. Binding sites with high affinity bound 50 nmoles of BSP per mg protein and those with low affinity bound 136 nmoles of BSP per mg protein. Dissociation constants calculated are 0.075 X 10^{-6} and 0.470 X 10^{-6} M respectively. As far as we know, this is the first study carried out on liver supernatant as a whole, from the point of view of BSP binding capacity.

Table 1 summarizes the results obtained from a set of five experiments. In each of them the two dissociation constants were calculated both for serum and liver supernatant taken from the same rat. The mean values ∓ the standard deviation, were calculated

Table 1
Dissociation constants (μM) of the two classes of binding sites for BSP of rat serum and rat liver supernate at 100 000 g.

Exp.	K_1 Serum	K_1 Supernate	K_2 Serum	K_2 Supernate
1	0.003	0.066	0.047	2.500
2	0.010	0.037	0.220	0.480
3	0.035	0.147	0.580	0.940
4	0.031	0.075	0.363	0.470
Mean	0.019±0.015	0.081±0.047	0.302±0.225	1.10±0.96
P	<0.05		<0.05	
Pool	0.015	0.150	0.250	0.890

for each constant. For both K_1 and K_2 the differences between serum and supernatant are statistically significant, with p values less than 0.05. Serum and supernatant derived from the two series of experiments were pooled, and the values of the two constants once again measured. One can see that a difference of one order of magnitude is found for K_1 and little less for K_2. The most interesting feature emerging from these data is the significant difference between dissociation constants of serum and liver supernatant, indicating a higher binding affinity for BSP of serum protein, than of the supernatant. For the high affinity binding capacities of the two fractions, we can calculate from the number of nmoles of BSP bound per mg of protein the total capacity for whole serum and liver supernatant. If we assume that in the rat there is approximately 10 ml of serum (containing 800 mg proteins) [11] and about 6 g of liver (containing 330 mg of soluble proteins) [12, 13], we obtain for the high affinity sites 48 nmoles of BSP bound to serum and 16.5 nmoles of BSP bound to liver supernatant. If we make the same calculation for the low affinity binding sites, we find 124 nmoles and 44.9 nmoles of BSP bound to serum and supernatnat proteins respectively. Hence the total amount of BSP bound to serum is 172 nmoles and 61.4 nmoles to supernatant. From these data we can note that the binding capacity of the proteins present on the outerside of the liver cell plasma membrane is about three-fold higher than that found on its inner side. The flux of organic anions from plasma to the liver by diffusion can be explained only by assuming a higher affinity of liver proteins than of serum proteins for the anion. From our data this not so, we find exactly the opposite: a higher affinity on the outer than on the inner side. Hence it is difficult on this basis to support the hypothesis that the transfer of organic anion from plasma into the liver occurs by nonionic diffusion across the plasma membrane. If, in fact, the total binding capacity and the affinity constant for BSP is higher in serum than in the supernatant, a negative gradient will be established across the liver cell plasma membrane. The simplest explanation of our data would be the existence of a carrier-mediated process at the level of the plasma membrane. The hypothesis of simple passive nonionic diffusion across the barrier is clearly insufficient to account for our findings. More direct evidence in favour of a carrier at the level of the plasma membrane is currently under investigation in our laboratory.

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