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INTERACTION OF BROMOSULFOPHTHALEIN WITH MITOCHONDRIAL MEMBRANES—UPTAKE OF BROMOSULFOPHTHALEIN AND EFFECT ON ANS-FLUORESCENCE

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Abstract—Bromosulfophthalein uptake by rat liver mitochondria is fast, but reversible and can reach >150 nmole/mg protein without lysis of mitochondria. There are no sets of characteristic binding sites with definite affinities, but affinity continuously decreases with increased binding. The decrease in binding affinity is ascribed to an increased negative surface charge resulting from insertion of the negatively charged bromosulfophthalein molecule into the hydrophilic–hydrophobic interphase of the membrane and rendering further binding of anionic molecules like bromosulfophthalein itself or ANS more difficult. This has been shown by the decrease of ANS-binding and fluorescence. The increase in surface charge on energization also results in decreased bromosulfophthalein binding. Although the amount of dye free in the matrix space, presumably is very small, the compound is possibly translocated and the amount bound to the inner side of the membrane may vary with the mitochondrial metabolic state.

Bromosulfophthalein is widely used in the clinical evaluation of hepatic function [1]. It is rapidly absorbed to plasma membranes and concentrated within isolated rat liver cells [2]. Occasional incidents in clinical liver function tests have raised the question about the cellular basis of toxicity. Killenberg and Hoppel [3] have shown that bromosulfophthalein inhibits mitochondrial state 3 respiration and incorporation of [^{32}P]phosphate. Very recently it has been shown that transport of anions, including phosphate, is inhibited by bromosulfophthalein [4].

In preliminary short communications [5,6], we have shown that besides inhibition of state 3 respiration, bromosulfophthalein affects uncoupled respiration and other mitochondrial functions. The present paper investigates the interaction of bromosulfophthalein with mitochondrial membranes and the effect on ANS-fluorescence.

In subsequent papers, kinetic analysis of respiratory inhibition of bromosulfophthalein [7] and effects on ion permeability [8] will be described and discussed on the basis of the interaction between the amphiphilic dye and membrane components.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats obtained from Ivanovas (Kisslegg, Germany) were fed a standard diet *ad lib.* and used at 200–300 g body weight. The following reagents were used: bromosulfophthalein from E. Merck (Darmstadt, Germany), [^{35}S]bromosulfophthalein (60 mCi/m-mole) from Radiochemical Centre via Amersham Buchler (Braunschweig, Germany); silicone oils AR 20 and AR 200 from Wacker Chemie (München, Germany); antimycin A from C. Boehringer (Mannheim, Germany); carbonyl cyanide *m*-chlorophenylhydrazone (CCP) from Calbiochem (Los Angeles, USA) and bovine serum albumin, 100% electrophoretically pure, from Behring–Werke (Marburg, Germany). All other reagents were purchased from E. Merck, Darmstadt, or C. Boehringer, Mannheim.

Isolation of mitochondria and preparation of submitochondrial particles. Liver mitochondria were isolated following the method of Klingenberg *et al.* [9]. The isolation medium contained 250 mM sucrose, 5 mM Tris and 0.5 mM EGTA. Submitochondrial particles were prepared from rat liver mitochondria by sonication in a MSE 20 KHz, 60 watt sonic oscillator according to Gregg [10]. Deviating from his procedure, there was a total sonication time of 5 min, sample cooling by an ice–salt mixture, centrifugation of the treated suspension at 20,000 *g* for 20 min and recentrifugation of the supernatant at 100,000 *g* for 30 min. The pellet was resuspended in 0.25 M sucrose medium. Protein concentrations were determined by a modified Biuret method [11].

Incubation of mitochondria. Incubations were started by diluting aliquots of the mitochondrial stock-suspension stored at 0° with incubation medium at 25°, unless otherwise stated. For further details, see legends to figures.

Measurement of bromosulfophthalein uptake. The uptake reaction was started by addition of 50 nCi [^{35}S]bromosulfophthalein and various concentrations of unlabelled bromosulfophthalein to 1 ml incubates. Mitochondria were separated from the incubation medium by centrifugal filtration [12]. For this purpose, 200-μl aliquots were layered onto 100-μl silicone oil made up of AR 20 and AR 200 5:1 in a centrifuge

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Abbreviations used: EGTA = Ethylenglycol-2-(2-aminoethyl)-tetracetic acid; CCP = Carbonyl cyanide *m*-chlorophenylhydrazone; ANS = 8-Anilinonaphthalene 1-sulfonic acid.

tube. On centrifugation in the Beckman 152 microfuge, the mitochondria were precipitated through the silicone layer into a deproteinizing layer of 50 μl 3M KOH within 10 sec. Radioactivity measurements of the supernatant and the mitochondrial sediment were performed in Bray's solution in a Berthold liquid scintillation counter.

Fluorescence measurements. Fluorescence of 8-anilinonaphthalene 1-sulfonic acid (ANS) was measured on excitation at 405 and 430 nm by emission at 470–3000 nm in 1-cm cuvettes using the fluorescence equipment supplied with the Eppendorf photometer.

RESULTS

Uptake of bromosulfophthalein by mitochondria. When bromosulfophthalein is added to a mitochondrial suspension, its free concentration is rapidly decreased by uptake.* Its time course is very fast (complete within 20 sec), and cannot be resolved by the centrifugal filtration technique. As can be seen from Table 1, the share of dye taken up by mitochondria depends on protein and dye concentration. Since the amounts of bromosulfophthalein which are taken up or free are of the same order of magnitude, different experiments are directly comparable only with equal concentrations of both dye and mitochondrial protein.

In Fig. 1, the results of a series of uptake experiments at constant mitochondrial concentrations are plotted according to Scatchard [14]. As indicated by the concave curvature, the affinity continuously decreases ("constants" may be deduced ranging from about $10^5\ M^{-1}$ to $10^4\ M^{-1}$).

The binding capacity of mitochondria is as high as >150 nmole/mg protein. Even under these high concentrations, binding is reversible, and the inner membrane of mitochondria is not really disrupted, since mitochondria are still centrifugable.

As can be seen from Fig. 2, uptake of bromosulfophthalein depends on the energetic state of the mitochondria. In the de-energized state, a little more of the dye is taken up.

Effect on ANS-fluorescence. When bromosulfophthalein is added to a mitochondrial suspension containing ANS, the ANS-fluorescence decreases. As shown in Fig. 3, above 5 nmole/mg protein, there is a logarithmic decrease with increasing bromosulfophthalein concentrations. The figure also shows that in sucrose medium the extent of fluorescence is much lower and is quenched only by higher concentrations of bromosulfophthalein.

It is demonstrated in Fig. 4 that the fluorescent dye is released from the mitochondria to the same extent as fluorescence decreases. Since diminution of ANS-fluorescence has been used as a measure of mitochondrial energization [15,16], it is of interest to compare the effects on mitochondria and on submitochondrial particles, which are "inside out" (Fig. 5). Though ANS-fluorescence in submitochondrial particles is increased on energization, it is also diminished with bromosulfophthalein addition, such as in mitochondria.

Table 1. Dependence on dye and protein concentration of bromosulfophthalein uptake

Mitochondrial protein (mg/ml)	0.37	0.75	1.9	3.7
Bromosulfophthalein Added (μM)	Taken up (%)			
5.5	45	64	79	80
13	35	56	79	78
19	28	49	70	76
32	22	37	63	74
52	16	28	57	70

Conditions were as in legend to Fig. 1, deviatory 55 nCi [^{35}S]bromosulfophthalein were added per incubate.

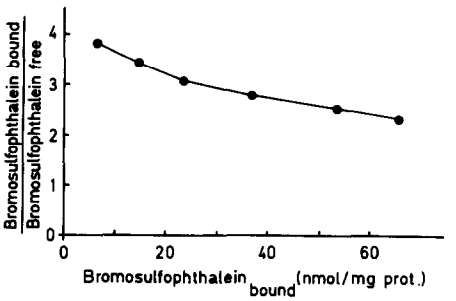

Fig. 1. Scatchard plot of the uptake of bromosulfophthalein by mitochondria. Rat liver mitochondria were diluted from stock suspension to a final concentration of 2.1 mg protein/ml in 1 ml incubation medium (140 mM KCl, 1.2 mM $MgSO_4$, 1 mM succinate, 0.7 mM Pi, pH 7.2) and incubated at 25°. One min after addition of 0.2 μCi [^{35}S]bromosulfophthalein of varying specific activity, 200-μl samples were withdrawn and mitochondria were separated from the incubation medium by centrifugal filtration to determine the free concentration (μM) of bromosulfophthalein and the amount of dye bound, by means of the specific activity. The results were plotted according to Scatchard.

DISCUSSION

On the basis of inhibition of anion transport, it has been assumed [4] that bromosulfophthalein is

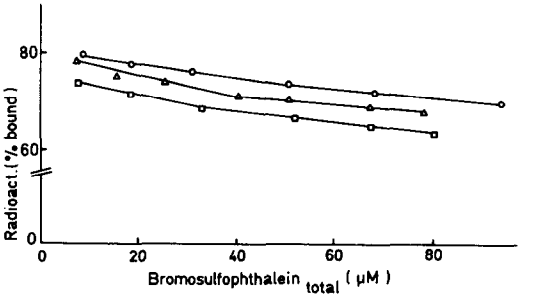

Fig. 2. Influence of the respiratory state on mitochondrial bromosulfophthalein uptake. Incubation conditions were as in Fig. 1, deviatory from a control (□), 1 μM CCP was added in one parallel incubation (△), and 5 μg antimycin A (○) in another.

*According to Azzone and Massari [13] the term "uptake" is used for disappearance from the supernatant without differentiating into transported or bound shares.

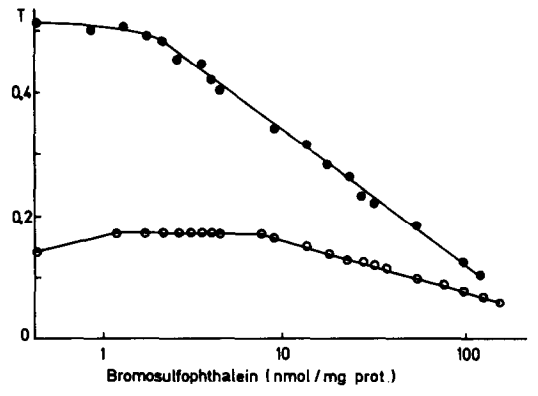

Fig. 3. Quenching by bromosulfophthalein of ANS-fluorescence. Mitochondria were suspended in a medium consisting either of 140 mM KCl (●) or of 250 mM sucrose (○) besides 5 mM Tris, 15 nmol ANS/mg protein, pH 7.4 at a concentration of 1.8 mg protein/ml at 25°. ANS-fluorescence was elicited at 405 and 430 nm and measured by emission at 470–3000 nm. The transmission was plotted against the logarithm of bromosulfophthalein concentration.

bound to the active site of translocating proteins. The concave curvature of the Scatchard plot (Fig. 1), however, did not reflect binding to a definite number of characteristic binding sites.

The affinity (association "constant" $K = 10^5\ M^{-1}$ to $10^4\ M^{-1}$), which gradually decreases, is comparable to the affinity reported for liver cell membranes ($K_1 = 1.8 \times 10^5\ M^{-1}$, $K_2 = 1.4 \times 10^4\ M^{-1}$) [2].

The uptake capacity of >150 nmole/mg protein approaches the order of magnitude of mitochondrial phospholipid content [17]. There is no doubt that under these conditions a considerable disorder of the phospholipid bilayer arises [7]. However, such destabilizing effects which usually result in the dissolution of biological membranes as observed for the plasma membrane at even lower concentrations [2, 18], seem to be partly overcome by protein:protein interactions in the mitochondrial membrane which raise the threshold for mitochondrial lysis.

It may be asked whether bromosulfophthalein is transported into the matrix space. Transport of these considerable amounts of a dibasic sulfonic acid should be a time-consuming process and should cause swelling. In contrast, uptake is so fast, that the time course cannot be measured with the methods used, and the volume is not increased, as is shown in a subsequent paper [8]. Since the mitochondrial membrane potential is inside negative [19, 20], it may be calculated that a dianion like bromosulfophthalein may be in the matrix space in concentrations of one one-thousandth of the outside concentration. Since it has been shown that ANS is able to penetrate the inner mitochondrial membrane [21, 22], it cannot be excluded that bromosulfophthalein too is transported across the membrane in very small amounts, which, however, are impossible to detect.

The uptake characteristics thus may reflect adsorption of the dye to the lipid–water interphase of the membrane in which it distributes at a constant ratio in analogy to a physical two-phase distribution. The aromatic ring system of the bromosulfophthalein molecule is thought of as being immersed into the hydrophobic part of the membrane as was analogously suggested for the ANS molecule [23], whereas the polar portion of the molecule, the sulfonic acid and phenolic groups, remain in the hydrophilic surface of the membrane. Thereby, the negative surface charge should be increased, leading to a diminished affinity for further bromosulfophthalein molecules, and resulting in characteristic interactions with other ionic compounds such as fluorescent dyes, as is shown in Fig. 3. ANS, which like bromosulfophthalein, has an aromatic sulfonic acid group, is also bound to the membrane interphase [21, 23, 24]. Since the quantum yield of ANS-fluorescence depends on the polarity

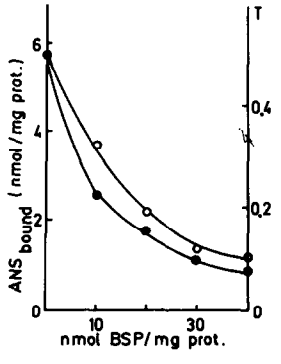

Fig. 4. Quenching of ANS-fluorescence and release of ANS by bromosulfophthalein. Mitochondria were incubated as in Fig. 1. Fifteen nmole of ANS/mg protein were added. The concentration of bromosulfophthalein was gradually increased and following each addition, ANS-fluorescence was measured as transmission (○). The amount of ANS bound to the membranes (●) was calculated from the free ANS concentration, which was obtained after addition of albumin to the supernatant of a centrifuged sample taken at different concentrations.

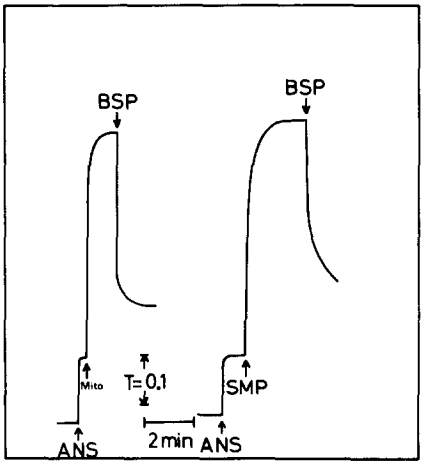

Fig. 5. Quenching of ANS-fluorescence with SMP and mitochondria by bromosulfophthalein. Submitochondrial particles (SMP) and mitochondria were suspended in KCl medium (1 mg protein/ml, 140 mM KCl, 5 mM Tris; pH 7.4). The concentration of ANS amounted to 15 μM. Bromosulfophthalein (BSP) was added at a concentration of 150 nmole/mg protein.

and viscosity of its vicinity in the membrane [25, 26], the dye is a suitable probe for changes in the surface charge density of mitochondria. In the energized state, where the surface charge is more negative [15,16,27,28], the ANS-fluorescence is lower when compared to the de-energized state [15,16]. This results from decreased binding affinity and lowered quantum yield [21, 29].

Displacement of ANS (Figs. 3, 4, 5) thus is due to the increase of negative surface charge of the membrane by bromosulfophthalein rather than to direct competition for the same binding sites. This is in agreement with the decrease of ANS-fluorescence in submitochondrial particles on binding of bromosulfophthalein. Thereby, the external ζ potential increases and thus overcomes the effect of energization which lowers the external surface charge. Analogously, an opposite effect of an increased ANS binding is observed after binding of positively charged biguanides to mitochondria [17]. Comparison of ANS-fluorescence in sucrose medium and in KCl medium fits well into this concept: In sucrose medium, where the effective charge density of the membrane is increased, less of both anions is bound. This has also been shown by Laperche and Oudea [4]. It results in a diminished extent of ANS-fluorescence and an increased concentration of bromosulfophthalein being demanded for quenching (Fig. 3).

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