Biochemical Pharmacology, Vol. 26, pp 825–832. Pergamon Press, 1977. Printed in Great Britain.

INTERACTION OF BROMOSULFOPHTHALEIN WITH MITOCHONDRIAL MEMBRANES: EFFECT ON ION MOVEMENTS

MICHAEL SCHWENK, REINHOLD BURR, HARTMUT BAUR and ERICH PFAFF*

Institute of Toxicology, University of Tübingen, D-7400 Tübingen, West Germany

(*Received* 27 *September* 1976; *accepted* 22 *November* 1976)

Abstract—The increase in negative surface charge density resulting from binding of the anionic dye bromosulfophthalein to mitochondrial membranes affects the movement of a variety of ions, depending on the dye concentration. At low concentrations, a sort of membrane-stabilization with decreased permeabilities is observed, whereas at high concentrations, a detergent like action occurs. (1). Acidification on respiration of mitochondrial suspensions is increased by very small concentrations (= 3 nmol/mg protein) of bromosulfophthalein. It reflects an increased transmembranal pH gradient. The effect is mainly due to inhibition of phosphate translocation. (2). Mitochondrial swelling experiments under specific conditions showed that transport of inorganic phosphate is inhibited in either direction. (3). Concentrations of > 100 nmol bromosulfophthalein/mg protein result in a membrane-destabilization. This is reflected in a release of Ca^{2+} ions and a subsequent increase in membrane permeability for succinate and inorganic phosphate. (4). All effects of bromosulfophthalein are freely reversible by the addition of albumin, to which the dye is bound with high affinity. (5). Similar effects can be observed with other compounds which are similar in structure, although none of those tried is effective in such small concentrations.

Bromosulfophthalein is a triphenylmethane dye which owes its strong anionic character to two sulfonic acid groups. Since the amphiphilic reagent is routinely used in clinical chemistry to test liver function, its interactions with biological structures have been intensely investigated. Strong binding to serum albumin [1] and interaction with the liver cell membrane [2] have been ascribed to its amphiphilic character. It is known that bromosulfophthalein interferes with mitochondrial functions [3, 4]. Very recently, Laperche and Oudea [5] have shown that transport of inorganic phosphate and other anions is inhibited by bromosulfophthalein. Since there are some inconsistencies in the results of these authors (see e.g. their Fig. 1 where ADP still stimulates respiration inhibited by bromosulfophthalein) which may be caused by difficulties in the experimental approach, the mechanisms of bromosulfophthalein action deserve further investigation, using other experimental techniques. Studies in this laboratory have shown that bromosulfophthalein results in a variety of membrane effects. It is of biochemical and toxicological interest to differentiate between the various mechanisms. In preceding papers, binding of bromosulfophthalein to mitochondrial membranes [6] and inhibition of respiration [7] have been described. It has been suggested that bromosulfophthalein acts on the activity of various respiratory components by the electrostatic effects of the increase in surface charge with binding. In the present paper, the concept of the changed surface charge, which may alter permeabilities of biological [8, 9] as well as artificial membranes [10–12], is applied to explain bromosulfophthalein effects on ion movement. This concerns moderate bromosulfophthalein concentrations, where membrane destabilizing effects are not yet apparent.

MATERIALS AND METHODS

Materials were essentially as described previously [6, 7], except for the following substances additionally used in the present experiments: ^3H_2O (250 μCi/ml), [^{14}C]carboxydextran (MW 75,000, 1.3 mCi/g), 5,5'-[2-^{14}C]dimethyloxazolidine-2,4-dione from NEN Chemicals, Dreieichenhain, Germany; [^{32}P]phosphate in HCl solution, carrier free from Amersham Buchler, Braunschweig, Germany; bromophenol blue, bromophenol red, bromthymol blue, phenol red, and fluorescein sodium from Merck, Darmstadt, Germany; auramine O and 4,5,6,7-tetrabromphenolphthalein from Eastman Kodak Co., Rochester, NY USA; rose bengal from Chroma Gesellschaft, Stuttgart, Germany, and sodium dodecylsulfate from Serva, Heidelberg, Germany. The best grades available were used without further purification.

The methods used have been described elsewhere: preparation of mitochondria [13], preparation of submitochondrial particles [14], determination of protein concentration [15], determination of adenine nucleotides (ATP by hexokinase method) [16], silicone centrifugation [17], measurement of adenine nucleotide translocation [18], measurement of proton movement [1], also for assay of ATPase, radioactivity measurement [cf. 6], measurement of swelling by turbidity measurements [cf. 19], and by direct vol. determination with ^3H_2O and [^{14}C]dextran [20].

RESULTS

Mitochondrial parameters demonstrating inhibition of phosphate transport by bromosulfophthalein. Figure

* To whom reprint requests should be addressed.

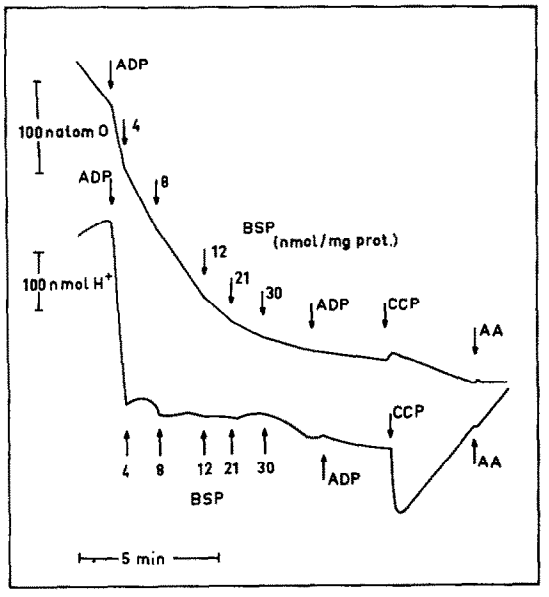


Fig. 1. Effect of bromosulfophthalein (BSP) on succinate respiration and accompanying proton movement. Mitochondria (1.3 mg mitochondrial protein/ml) were suspended in KCl medium consisting of 140 mM KCl, 1 mM $MgSO_4$, 0.8 mM P_i, 10 mM succinate and 4 μM rotenone at pH 7.1 and 25°. Additions were made as indicated in the figure: 2 mM ADP, bromosulfophthalein in consecutive additions, 1 μM CCP and 3 μg antimycin A/ml. Respiration and concomitant proton movements were measured synchronously, but in parallel incubations.

Table 1. Inhibition by bromosulfophthalein (BSP) of the mitochondrial ATPase

Subsequent additions	Oxygen uptake (natom O × min^{-1}) × mg $protein^{-1}$)	Release of protons (nmol H^+ × min^{-1} × mg $protein^{-1}$)
None	18	−3
CCP	82	−50
ATP	82	175
BSP (nmol/mg prot)		
10	55	37
20	51	25
30	46	15
40	35	11
Succinate, 4 mM	16	14

Mitochondria (1.2 mg mitochondrial protein) were suspended in 1 ml medium made up of 140 mM KCl, 10 mM Tris, 5 mM pyruvate, 0.5 mM malate, 1.2 mM $MgSO_4$ at pH 7.2 and 25°. Additions were made subsequently. ATPase activity was elicited by addition of 1 μM CCP prior to 4 mM ATP and was monitored by proton release. The concomitant oxygen consumption was followed.

1 shows the effects of bromosulfophthalein on oxygen consumption and on the concomitant proton movements in state 3. The increase in oxygen consumption on addition of ADP corresponds to a respiratory control ratio of 3.2. The concomitant proton uptake upon ATP synthesis amounts to 5.2 H^+/O. On addition of small amounts of bromosulfophthalein (4 nmol/mg protein), respiratory and pH traces immediately return to state 4 conditions (the gradual inhibition of the remaining respiration by further bromosulfophthalein additions has been considered in a preceding paper [7]).

On uncoupling, ATP hydrolysis is stimulated, resulting in H^+-ejection. At pH 7.2, about 0.8 H^+ is liberated in the hydrolysis reaction itself. Since the mitochondrial ATPase is not accessible from the extramitochondrial space, the observed net proton efflux of 1.3 protons results from ATP splitting and phosphate release and, in addition, from differences in pK values of ATP taken up and ADP released [21]. Phosphate efflux makes the biggest contribution to proton liberation [21]. This may be expected to be abolished, if phosphate is retained in the matrix space. Table 1 demonstrates that on addition of bromosulfophthalein, acidification is diminished. As shown below, direct inhibition of the ATPase at these low concentrations could be ruled out. Here, inhibition of phosphate transport out of the mitochondria may be the cause of this bromosulfophthalein effect.

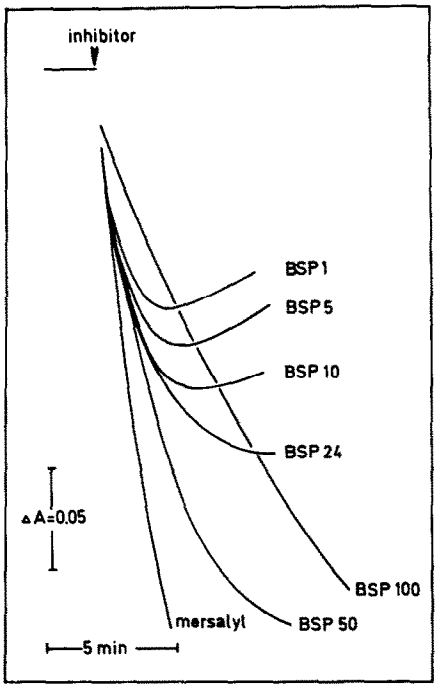
A comparison of the degree of inhibition imposed by bromosulfophthalein on oxygen consumption in state 3 and on proton release in ATP hydrolysis, respectively, is given in Table 2. Nearly identical I_{50} values suggest that both effects are caused by a common mechanism. This appears to be phosphate translocation since it is involved in both reactions, either as uptake (as in state 3 respiration), or as release (as in the ATPase reaction). Inhibition in both cases is independent of the substrate used.

When the mitochondrial ATPase is stimulated by carbonyl cyanide *m*-chlorophenylhydrazone (CCP) and ATP, the hydrolysis products leave the matrix space via the adenine nucleotide translocase and the phosphate carrier. If, however, the phosphate carrier is inhibited, phosphate remains in the matrix space, causing intensive swelling [19]. As is seen from Fig. 2a, swelling is induced by bromosulfophthalein. Its velocity increases with concentration. The maximal extent of swelling is comparable to that caused by mersalyl. This demonstrates again that not only influx, but also efflux of inorganic phosphate is inhibited by bromosulfophthalein. This inhibition can be

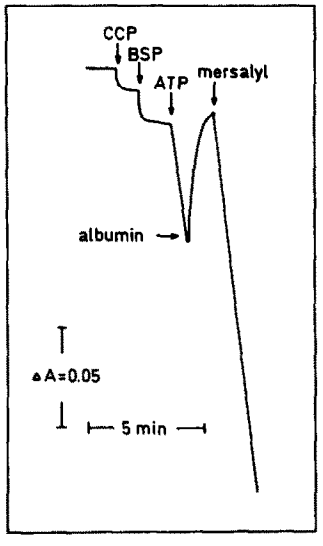
Table 2. Comparison of I_{50} values on bromosulfophthalein inhibition of active respiration and ATP hydrolysis

Substrate, mM	State 3 respiration	ATP-hydrolysis
	I_{50} (nmol/mg protein)	
Succinate		
2	4	3.5
4	5.5	3.5
Pyruvate		
2	3	3.5
4	4	3
β-Hydroxybutyrate		
2	3.5	4.5
4	4	4.5
Glycerol-phosphate		
4	3	4

The experimental procedure was as in the experiment of Table 1. I_{50} values were derived as mean values from three independent measurements.



(a)

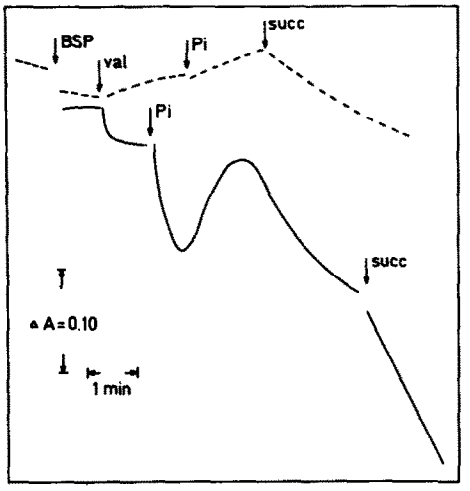


(b)

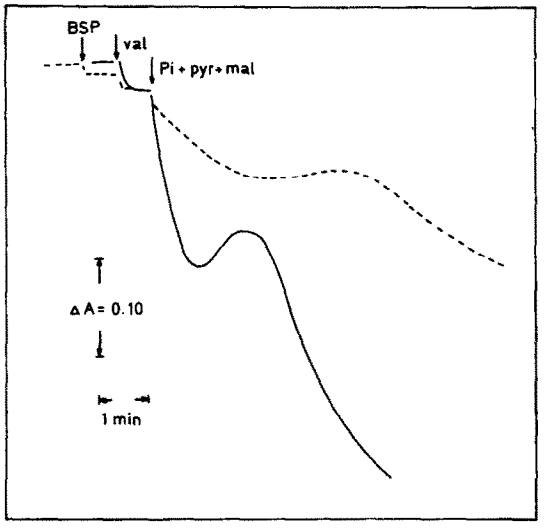
Fig. 2 (a, b). Inhibition by bromosulfophthalein (BSP) of release of mitochondrial P_i. Mitochondria were suspended in sucrose medium containing 220 mM sucrose, 15 mM KCl, 0.1 mM $MgSO_4$, 4 mM triethanolamine buffer at pH 7.4 and 25° to a final concentration of 0.5 mg mitochondrial protein/ml. The optical density was followed at 546 nm in a 10 mm cuvette. ATPase activity was elicited by 1 μM CCP in the presence of 1 mM external ATP. Bromosulfophthalein concentrations in Figure 2a are given in nmol/mg protein; mersalyl concentration was 100 μM. In Figure 2b, 10 nmol bromosulfophthalein/mg protein and 0.5 mg albumin/ml were added.

released by addition of albumin, but then is reimposed by mersalyl (Fig. 2b).

Mitochondrial swelling in the presence of substrate and valinomycin plus potassium ions is induced by addition of phosphate and shows oscillatory cycles. It can be monitored photometrically [22, 23]. Volume oscillations after phosphate addition [24–26] are quenched by bromosulfophthalein (Fig. 3a). As shown in Fig. 3b, in the presence of substrate, there is a residual oscillation with smaller amplitude and increased period. Similar effects as with pyruvate plus malate are seen in the presence of other substrates. Again, these findings can be explained by inhibition of influx of phosphate by bromosulfophthalein.



(a)



(b)

Fig. 3 (a, b). Inhibition by bromosulfophthalein of the cyclic swelling-shrinking process. Incubation conditions and monitoring of vol. changes were as in the legend to Figure 2. Additions were 2.5 ng valinomycin/ml, 5 mM succinate or pyruvate, 0.5 mM malate, 2.5 mM P_i. Figure 3a shows vol. oscillations after phosphate; Figure 3b after addition of phosphate and substrate with and without 20 nmol bromosulfophthalein/mg protein.

The results indicate that the adenine nucleotide influx and the mitochondrial ATPase are not markedly affected by bromosulfophthalein in the concentrations used. The velocity of swelling is reduced only above 50 nmol bromosulfophthalein/mg, which may be due to the inhibition of either the ATPase or the adenine nucleotide translocation at these high bromosulfophthalein concentrations.

Inhibition of adenine nucleotide translocase and ATPase. It is not clear how Laperche and Oudea [5] calculated the K_i values of 4.4–1.75 nmol/mg protein for the inhibitory action of bromosulfophthalein on adenine nucleotide translocation. Our own experiments using the inhibitor stop method [18] gave a value for half maximal inhibition of ADP[^{3}H]-ATP exchange of about 35 nmol bromosulfophthalein/mg protein.

In addition, high concentrations of bromosulfophthalein seem to inhibit the ATPase reaction as was seen by use of submitochondrial particles, where no permeation step interferes with the ATPase reactions. The hydrolysis of ATP by the CCP stimulated ATPase, as measured by acidification of the suspension, was inhibited with a half maximal effect at 45 nmol bromosulfophthalein/mg protein.

Effect of bromosulfophthalein on proton movements. Mitochondria respiring in state 4 build up a pH gradient by transmembranal proton movement [27, 28]. In unbuffered incubations, this can be measured as acidification of the medium. Figure 4 shows effects

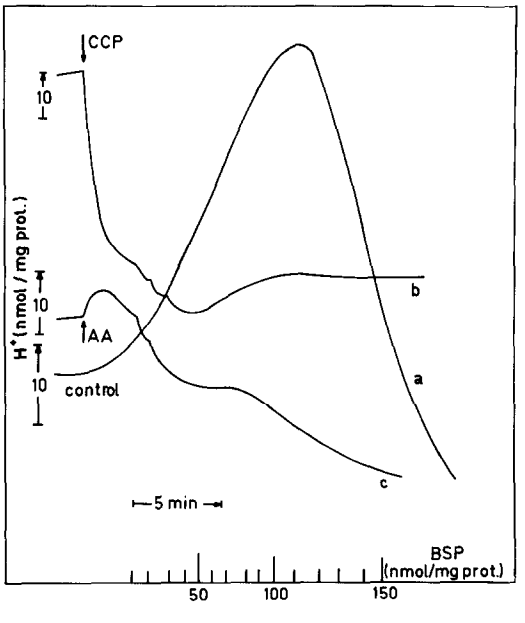


Fig. 4. Proton movements on bromosulfophthalein addition to mitochondria. Mitochondria (5 mg mitochondrial protein) were suspended in 2.5 ml KCl medium consisting of 150 mM KCl, 0.5 mM P_i, 1 mM $MgSO_4$ and 2 mM β-hydroxybutyrate at 25°. pH changes were followed with a glass electrode and calibrated by known amounts of HCl. Bromosulfophthalein was repeatedly added in amounts of 10 nmol/mg protein at a time as indicated by the strokes at the base line of the figure. One μM CCP was added in experiment b; 4 μg antimycin A/ml in experiment c. For direct comparison of the experiments, the different buffering capacities of the mitochondria in the various states have to be considered.

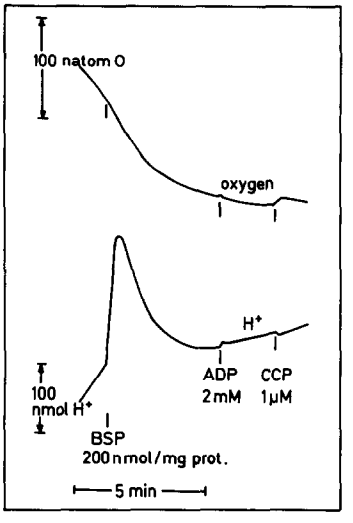


Fig. 5. Respiration and proton movements in response to a single high dose of bromosulfophthalein. Mitochondria were suspended in KCl medium (150 mM KCl, 1 mM $MgSO_4$, 0.8 mM P_i, 10 mM pyruvate, 1 mM malate at pH 7.1 and 20°) to a final concentration of 1.3 mg protein/ml. In separate incubations, pH movement and respiration were synchronously followed on addition of 200 nmol bromosulfophthalein/mg protein.

of bromosulfophthalein on proton movements under various conditions. The acidification in state 4 (curve a) is increased by bromosulfophthalein. After addition of a total amount of 80–100 nmol bromosulfophthalein/mg protein, there is a short latency period, then the pH trend is reversed, and the suspension becomes even more alkaline than at the beginning of respiration.

As can be seen from a comparison with curve b, this additional release of hydroxyl ions is almost identical to that caused by the uncoupler CCP. On the other hand, if bromosulfophthalein is added after the reequilibration of protons across the membrane in the presence of CCP (curve b), it initially leads to a slightly increased alkalinization with a subsequent slight acidification. Under the conditions of the experiment of Fig. 4, inhibition of respiration by antimycin A (curve c) also prevents acidification by bromosulfophthalein.

The effect of a single very high dose of bromosulfophthalein (200 nmol/mg protein) is shown in Fig. 5. The initial acidification of the extramitochondrial space is accompanied by a transient increase in oxygen consumption which is not observed if bromosulfophthalein is added in small amounts. The observed proton extrusion suggests that the increased respiration is employed to transport protons actively from the matrix space to the extramitochondrial space in order to establish the new transmembranal pH gradient.

The gradual inhibition of oxygen consumption after about 90 sec is accompanied by an alkalinization of the medium. Thereafter, ADP and CCP have no more effect on respiration or proton movements, indicating membrane leakage.

Movements of ions. The inhibitory effect of bromosulfophthalein on substrate dehydrogenases [7] might

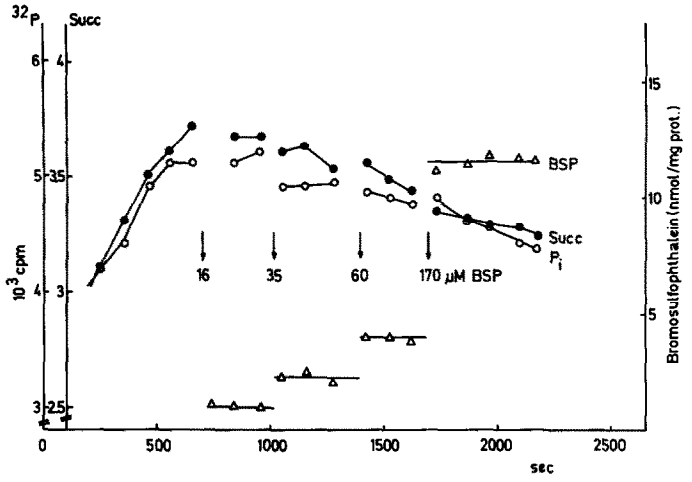


Fig. 6. Release of succinate and P_i by bromosulfophthalein. Mitochondria (20 mg mitochondrial protein) were suspended in 4.2 ml of 150 mM KCl medium with 5 mM Tris, pH 7.2 and 25 . At zero time incubation was started by addition of 12 μM [^{14}C]succinate, 1 μM malonate and 12 μM [^{32}P]P_i. Uptake was followed by centrifugal filtration. At each time indicated, bromosulfophthalein additions were made. Total concentrations added are given. After each addition, the mitochondrial content of bromosulfophthalein, succinate and [^{32}P]P_i was determined.

be caused by competition with other anions for translocation. If this were true, one should observe efflux of succinate or phosphate on additon of bromosulfophthalein. To test this hypothesis, mitochondria were incubated with [^{14}C]succinate in the presence of malonate. Addition of bromosulfophthalein stops uptake of [^{14}C]succinate and [^{32}P]inorganic phosphate (P_i), but at low amounts, intramitochondrial succinate and phosphate remain constant. With high bromosulfophthalein additions, there is a gradual increase of the rate of efflux (Fig. 6).

Likewise, [^{32}P]P_i fluxes were followed, together with the pH_0, in the medium, and dimethyloxazolidine-dione (DMO) distribution ratios. DMO is a weak acid and distributes between compartments according to the Δ pH. Figure 7 shows that low concentrations of bromosulfophthalein lead to the acidification of the suspension, already described, and an alkalinization of the matrix. The pH change in the matrix exceeds that of the medium. Thus, the distribution of DMO indicates that this effect is not merely a membrane effect, but that protons are actually transferred from the matrix to the extramitochondrial space. Again, the phosphate distribution remains constant at these concentrations.

High concentrations of bromosulfophthalein (120 nmol/mg protein) lead to an efflux of P_i and to a sudden increase of extramitochondrial pH while intramitochondrial pH continues to increase for a short time before it also declines. This appears as a specific release of P_i as phosphoric acid or in symport with protons before further additions of bromosulfophthalein render the membrane unspecifically permeable. Thus, as shown in Fig. 8, at 80 nmol bromosulfophthalein/mg/protein, K^+ leaks out of the mitochondria. This increased unspecific permeability seems to be caused by the release of Ca^{2+} by bromosulfophthalein, which plays an important role in the regulation of permeabilities of the membrane [cf. 29]. Figure 9 indicates that the release of ^{45}Ca into the supernatant precedes the influence on general permeability of the mitochondrial membrane.

The labilization of the membrane by high amounts of bromosulfophthalein is not, however, an irrevers-

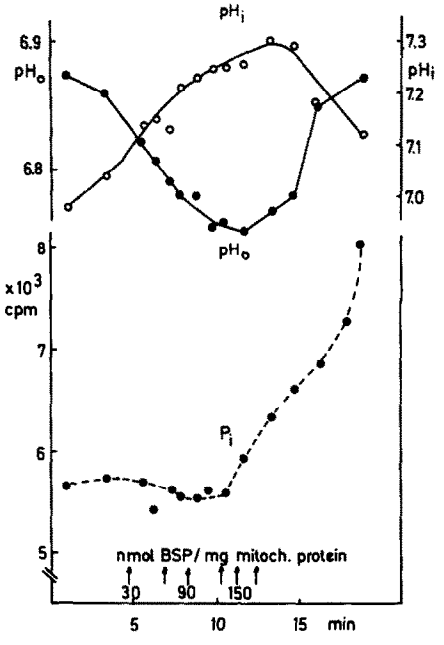


Fig. 7. Proton movement, pH_0, mitochondrial pH_i, and release of P_i on bromosulfophthalein addition. Mitochondria (12 mg mitochondrial protein) were incubated in 3.4 ml KCl medium (150 mM KCl, 1 mM Tris buffer, 1.2 mM $MgSO_4$). At zero time, 3.4 μmol [^{3}H]DMO (0.45 μCi) and 0.8 μmol [^{32}P]P_i (0.5 μCi) were added. The pH_0 of the medium was continuously recorded. 200 μl samples were subjected to centrifugal filtration to determine extramitochondrial P_i and mitochondrial and medium DMO concentrations from which pH was calculated. At each time indicated, 30 nmol bromosulfophthalein/mg protein was added.

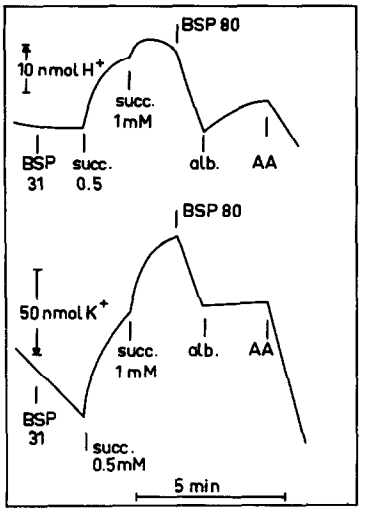


Fig. 8. Simultaneous registration of proton and K^+ movement on bromosulfophthalein addition. Mitochondria (4 mg mitochondrial protein) were suspended in 2.5 ml KCl medium (140 mM KCl, 10 mM sucrose, 1.2 mM $MgSO_4$, 1 mM triethanolamine buffer, 5 ng valinomycin). Simultaneously, there was measured in the medium the pH by a pH electrode and the K^+ concentration by a highly sensitive potassium electrode (Philips IS560K). Additions were 5 mM pyruvate, 0.5 mM malate, 1 mM P_i, 4 μM rotenone, 31 and 80 nmol bromosulfophthalein/mg protein, respectively, 1 mM succinate, 2 mg albumin, 3 μg antimycin A.

ible process. K^+ and P_i release are still partially reversible with albumin even if the gradients of these ions completely disappear across the membrane. Thus, we observed a reuptake of 80 per cent of the released Ca^{2+} and 23 per cent of the released P_i on additon of 17 μM bovine serum albumin to a mitochondrial suspension, to which antimycin A was added prior to a total of 80 μM bromosulfophthalein.

Transport of bromosulfophthalein into the matrix space. After it has been demonstrated that bromosulfophthalein does not enter the mitochondria in any measurable amount via the dicarboxylate carrier, the question arises if bromosulfophthalein enters the matrix space at all. If it passed through the inner membrane in symport with a counterion, osmotic swelling should be observed. The matrix vol., however, as measured with HTO- and [^{14}C]dextran, was not altered by bromosulfophthalein. Only at concentrations where the membrane is disorganized is there a rapid decrease in turbidity.

If bromosulfophthalein permeated as undissociated acid, it would cause alkalinization of the medium. Acidification is observed, however.

Inhibitory effects of bromosulfophthalein analogues. If its amphiphilic nature causes the effects of bromosulfophthalein [6], it can be expected that compounds with similar amphiphilic character, such as other triphenylmethane dyes with sulfonic acid groups, will exert similar effects. To study this possibility, we tested the effect of bromthymolblue, bromophenolblue, bromophenolred, phenolred, bengal rose, fluorescein, 1-anilino-naphthalene-8-sulfonic acid (ANS), sodium dodecylsulfate (SDS), taurocholate and tetrabromophenolphthalein on phosphate transport and on uncoupled respiration (Table 3). It can be seen that within this group of substances, bromosulfophthalein has by far the strongest inhibitory effect in both mitochondrial functions.

DISCUSSION

It has been shown that binding of bromosulfophthalein to mitochondrial membranes [6] not only results in inhibition of respiration [7], but also affects various membrane permeability qualities.

Exchange of bromosulfophthalein for succinate or inorganic phosphate could not be observed (Fig. 6). In addition, one may calculate if bromosulfophthalein permeated as a dianion, only about one-ten thousandth of the external concentration could be expected to be in the matrix space, assuming a membrane potential of e.g. 100 mV. Thus, it seems to be

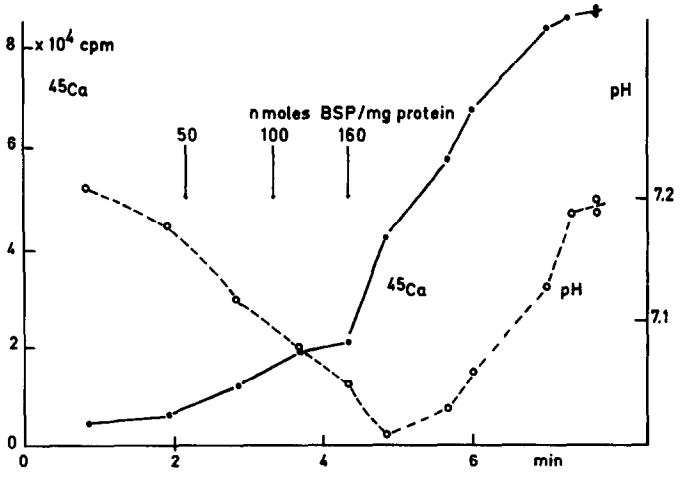


Fig. 9. Simultaneous measurement of calcium release and proton movement on bromosulfophthalein addition. Mitochondria (5 mg mitochondrial protein) were suspended in 3 ml KCl medium (as in Fig. 6); 0.4 μCi ^{45}Ca were added. Total calcium was about 15 nmol/mg protein. The pH_0 was registered. Samples were withdrawn and centrifuged for measurement of ^{45}Ca in the supernatant.

Table 3. Inhibition of respiration by amphiphilic anions

Substance	Respiration	
	State 3	Uncoupled
Bromosulfophthalein	5.5	9.5
Bromthymolblue	22	20
Bromophenolblue	26	32
SDS	40	—
Bengalrose	48	—
Phenolrot	66	70
Bromphenolrot	82	70
Fluorescein	—	—
ANS	—	—
Tetrabromphenolphthalein	—	—
Taurocholate	—	—

The table gives I_{50} values for the inhibition of respiration by various amphiphilic anions in the presence of 4 mM succinate. The substances are arranged in order according ot their effectiveness. A dash means no measurable effect.

reasonable to discuss all the effects on mitochondrial functions from the view of increased negative surface charge density [6], triggering permeabilities of the membrane and enzyme activities [7]. This is in agreement with the fact that most of the enzymes are located on the inner surface of the inner mitochondrial membrane [30–32].

The effects at small concentrations (< 50 nmol/mg protein) may be summarized as membrane stabilization. Most impressive is the inhibition of phosphate translocation. Kinetics of respiratory inhibition [7] suggest that two molecules are acting as an inhibitory unity at or in the vicinity of the phosphate carrier.

The fact that not only uptake (Fig. 3) but also efflux (Fig. 2) of phosphate is inhibited by bromosulfophthalein suggests that it is not only the access and binding of the anionic phosphate that is inhibited by the increased external negative surface charge, but that the phosphate translocase is immobilized, preventing conformational changes necessary for translocation [cf. 33, 34].

The inhibitory effect on phosphate translocation caused by bromosulfophthalein is even stronger than that caused by the mercurial compound, mersalyl (Fig. 2a). Likewise, at small concentrations of bromosulfophthalein, increased proton extrusion (Fig. 4, 5) and increased transmembranal proton gradient (Fig. 7) are observed. This effect resembles energization by respiration in also having increased external surface potential of the mitochondrial membrane [27]. But while in bromosulfophthalein action dissociated sulfonic acid groups are involved, in respiratory energization the charges are derived from dissociating groups of membrane components. (The pH effect of bromosulfophthalein is, however, in great part due to inhibition of phosphate translocation.) Effects of similar substances [35] reported in abstract form have been discussed analogously.

In contrast to membrane stabilization at small concentrations, high concentrations (> 100 nmol/mg protein) result in a membrane labilization. Release of Ca^{2+} ions is followed by increased permeability for inorganic phosphate (Fig. 9) and breakdown of transmembranal pH gradient (Fig. 7). The permeability changes are gradual, as can also be deduced from the dissociation of changes of the Δ pH from Ca^{2+} release and P_i movement (Fig. 8, 9).

These effects may be described as decreased packing of the phospholipid structure [cf. 32] on bromosulfophthalein binding, from which inhibition of ATPase reaction (Table 2) and of adenine nucleotide transport may result.

Bromosulfophthalein is a very potent inhibitor of mitochondrial functions. All observed effects are, at least in part, freely reversible by the addition of albumin (Fig. 2b) to which the dye is bound [2]. Thus, the compound offers itself as a useful tool in mitochondrial research.

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