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INTERACTION OF BROMOSULFOPHTHALEIN WITH MITOCHONDRIAL MEMBRANES—INHIBITION OF RESPIRATION

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Abstract—Bromosulfophthalein reversibly inhibits mitochondrial respiration. Most sensitive is state 3 respiration (K_i about 3 nmole/mg protein independent of substrate). At higher concentrations (20–100 nmole/mg protein) state 4 respiration and uncoupled respiration are also inhibited. This inhibition is substrate dependent. With succinate, inhibition appears to be noncompetitive at low concentrations and competitive above 1 mM succinate (half-maximal inhibition at 9–17 nmole/mg protein, dependent on succinate concentration). Substrate permeation seems to be not the only sensitive step in oxidation, as is deduced from similar results obtained on glycerol phosphate respiration. By use of artificial electron shunts and by difference spectroscopy, individual dehydrogenases have been made probable as the site of bromosulfophthalein action. It is suggested that bromosulfophthalein acts via the electrostatic effects of the increased negative surface charge, making dehydrogenases less accessible for their substrates.

The preceding paper [1] has shown that the amphiphilic dye bromosulfophthalein is bound in appreciable amounts to mitochondrial membranes. The effects on ANS-fluorescence indicated that the negative surface charge density is thereby increased. Killenberg and Hoppel [2] have shown that bromosulfophthalein inhibits state 3 mitochondrial respiration.

This paper presents kinetic analysis of respiratory inhibition and shows that in addition to state 3 respiration, mitochondrial respiration is inhibited also under other conditions. The various inhibitory effects at varying concentrations of inhibitor and conditions are discussed on the basis of increased negative surface charge density.

MATERIALS AND METHODS

Materials and methods are described in the preceding paper [1]. In the present paper, the following additional techniques are applied.

Measurement of respiration. Uptake of oxygen was measured with a Clark oxygen electrode at 25°. The reaction was started by addition of 50 μl of mitochondrial stocksuspension to 1.1 ml medium, kept at 25°.

Difference spectra. Difference spectra of mitochondrial suspensions were taken with an Aminco Chance spectrophotometer. For experimental details, see Fig. 9 and [3].

RESULTS

Inhibition of state 3 respiration

Recently it has been shown that transport of anions across the inner mitochondrial membrane is inhibited by bromosulfophthalein [4]. The question thus arises whether the oxidation of substrates which do not need to pass through the inner mitochondrial membrane also is inhibited. This is the case for glycerophosphate, since glycerol phosphate dehydrogenase (EC 1.1.99.5) is known to be accessible from the outer surface of the mitochondrial inner membrane [5]. As demonstrated in Fig. 1, state 3 respiration with glycerol phosphate as substrate is inhibited by bromosulfophthalein. The inhibition is of a mixed competitive–noncompetitive type. The Dixon plot (Fig. 2) allows

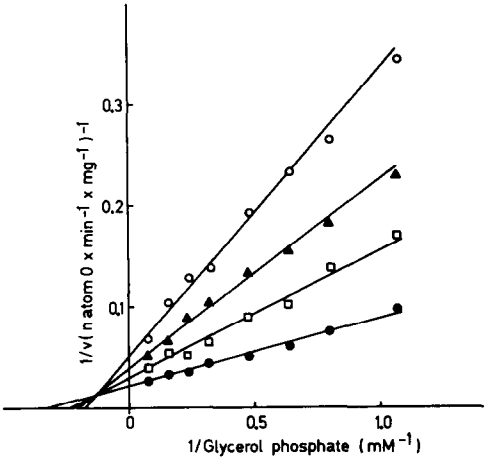

Fig. 1. Lineweaver–Burk plot of state 3 glycerol phosphate respiration at various bromosulfophthalein concentrations. Mitochondria (3.2 mg protein) were incubated at 26° in 1 ml sucrose medium containing 250 mM sucrose, 4 mM Tris, 1 mM KH_2PO_4, 3 μM rotenone, 1 mM malonate, 4 mM $MgSO_4$, and 0.6 mM ADP. For induction of glycerol phosphate oxidase the rats were injected with 200 μg triiodothyronine i.p. 2 days prior to sacrifice. Bromosulfophthalein concentrations in nmole/mg protein: control = none (●), 2.5 (□), 5 (▲), 8.4 (○).

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Abbreviations used: EGTA = Ethylenglycol-2-(2-aminoethyl)-tetra acetic acid; CCP = Carbonyl cyanide *m*-chlorophenylhydrazone; Tris = Tri(hydroxymethyl) amino methane.

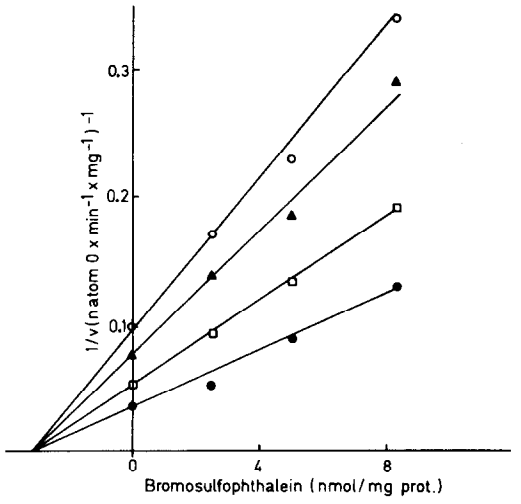

Fig. 2. Dixon plot of state 3 glycerol phosphate respiration in the presence of bromosulfophthalein. Conditions were as in Fig. 1. The $1/v$ values obtained from this figure at various glycerol phosphate concentrations are plotted against the bromosulfophthalein concentration. The respective glycerol phosphate concentrations were: 0.9 mM (○), 1.25 mM (▲), 2.1 mM (□), and 4.2 mM (●).

us to calculate a K_i = 3.2 nmole bromosulfophthalein/mg protein. A similar type of inhibition was found for state 3 respiration with succinate as substrate. The resulting Dixon plot was nonlinear with an upward bend. The effects on relative rates of oxygen consumption are identical for both substrates used.

Comparison of inhibition by bromosulfophthalein of state 3 respiration with the action of inhibitors of oxidative phosphorylation

The above results are in line with the finding that oxidative phosphorylation is inhibited by bromosulfophthalein [2]. To further characterize the mode of inhibition on phosphorylation, the bromosulfophthalein action is compared with that of some well-known inhibitors of oxidative phosphorylation (Table 1). Bromosulfophthalein is more effective in lower concentrations than the other inhibitors used. Dependent on the inhibitor, the inhibition is either completely or partly abolished on addition of the uncoupler CCP. Bromosulfophthalein resembles mersalyl in being partly inhibitory even in the uncoupled state.

Inhibition of uncoupled respiration

Inhibition of mitochondrial respiration is not restricted to state 3 respiration. Another effect occurring at higher dye concentrations is most prominent at maximal respiratory rates. This can best be demonstrated in the uncoupled state.

(a) *Succinate as substrate.* Figure 3 shows, in the double reciprocal plot according to Lineweaver–Burk, kinetics of inhibition of uncoupled mitochondria by bromosulfophthalein with succinate as substrate. The curves have a break at about 1 mM succinate, which is most prominent with high bromosulfophthalein concentrations. Breaks in the Lineweaver–Burk plot have similarly been found for malate dehydrogenase (l-malate: NAD oxidoreductase; EC 1.1.1.37) [6] and for glutamic acid dehydrogenase (l-glutamate: NAD oxidoreductase; EC 1.4.1.2) [7] and have been interpreted as substrate activation. At substrate concentrations above 1 mM, inhibition seems to be competitive with the characteristic constants: V = 200 natom O min^{-1} mg^{-1} protein and K_m = 0.7–25 mM succinate. The K_m for succinate increases proportionally to the square of the bromosulfophthalein concentration (Fig. 4). At lower substrate concentrations there seems to be a rather noncompetitive type of inhibition. A Dixon plot of these data (Fig. 5) is not linear. The K_i value can only tentatively be read from this plot to be about 7 nmole/mg protein, whereas half-maximal inhibition is found with 9–17 nmole bromosulfophthalein/mg protein, dependent on substrate concentration.

Table 1. Comparison of the effects of bromosulfophthalein and mitochondrial inhibitors on respiratory rates

Inhibitor	Bromosulfophthalein	Atractyloside	Oligomycin	Mersalyl
Subsequent additions	Respiratory rate (natom O min^{-1} mg^{-1} protein)			
4 mM Succinate	45	44	41	44
2 mM ADP	182	176	177	180
Inhibitor				
1st addition	123	55	41	92
2nd addition	84	33	39	35
1 μM CCP	143	207	216	144
4 mM β-Hydroxybutyrate	16	15	13	14
2 mM ADP	77	77	76	77
Inhibitor				
1st addition	56	14	13	33
2nd addition	38	10	13	13
1 μM CCP	60	70	68	59

Mitochondria (2.5 mg protein/ml) were suspended in KCl medium (140 mM KCl, 1.5 mM P_i, 1.5 mM $MgSO_4$, 20 mM Tris, pH 7.3) at 25°. Additions were subsequently given as indicated. The concentrations of the respective inhibitors were: bromosulfophthalein, first addition, 4 nmole/mg protein = 10 μM; second addition 8 nmole/mg protein = 20 μM; actractyloside, first addition 50 μM; second addition 100 μM; oligomycin, first addition 10 μg/ml; second addition 20 μg/ml; mersalyl, first addition 40 μM; second addition 80 μM.

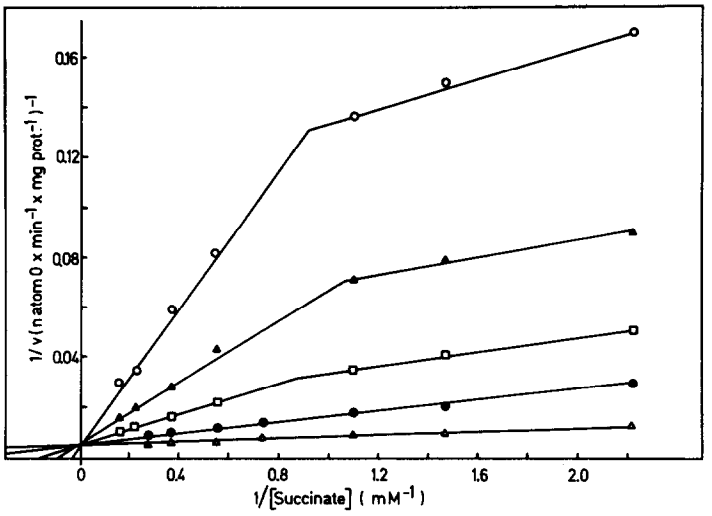

Fig. 3. Lineweaver–Burk plot of succinate respiration in the uncoupled state at various bromosulfophthalein concentrations. Mitochondria (2.2 mg protein) were incubated at 26° in sucrose medium consisting of 250 mM sucrose, 10 mM KCl, 0.7 mM P_i, 4 μM rotenone, 1 μM CCP, at pH 7.2. Bromosulfophthalein concentrations in nmole/mg protein: control = none (△), 10.5 (●), 21 (□), 31 (▲), and 53 (○).

(b) *Glycerol phosphate as substrate.* The question arises whether inhibition of uncoupled respiration results from effects on substrate permeation. Oxidation of glycerol phosphate is independent from substrate transport, but as is shown in Fig. 6, glycerophosphate respiration too is competitively inhibited by bromosulfophthalein. Half-maximal inhibition is attained with about 14 nmole bromosulfophthalein/mg protein. The K_m for glycerol phosphate, similar to that for succinate, also increases proportionally to the square of the bromosulfophthalein concentration. Thus, the site of inhibition is not the translocation of substrates, but a step in oxidation common to both substrates.

(c) *Inhibition of dehydrogenases.* To localize the site of inhibition in uncoupled respiration, various segments of the electron transport chain were separately investigated by the use of artificial electron acceptors: with the exception of the dehydrogenases, all other reaction steps in the respiratory chain appear to be unaffected. The dehydrogenases were measured by use of methylene blue as artificial electron acceptor [8] and its ability to directly react with molecular oxygen. In Fig. 7, the effect of bromosulfophthalein on oxygen uptake of uncoupled mitochondria in the presence of various substrates is compared to that on oxygen uptake of their respective dehydrogenases in the presence of respiratory inhibitors and methylene blue.

Using pyruvate as substrate, respiration and dehydrogenase reaction are very slow and only slightly inhibited by bromosulfophthalein. With β-hydroxybutyrate as substrate, both reactions are faster and

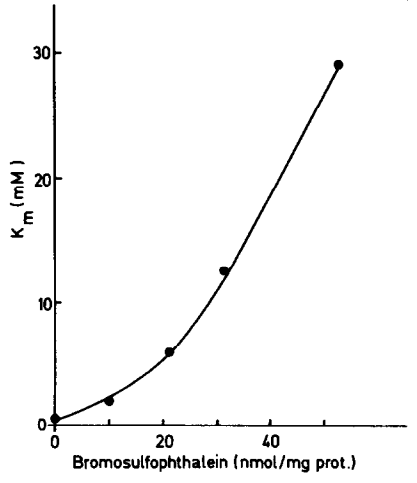

Fig. 4. Influence by bromosulfophthalein on the K_m of succinate respiration. K_m values were deduced from the data of Fig. 3 and plotted as a function of bromosulfophthalein concentration.

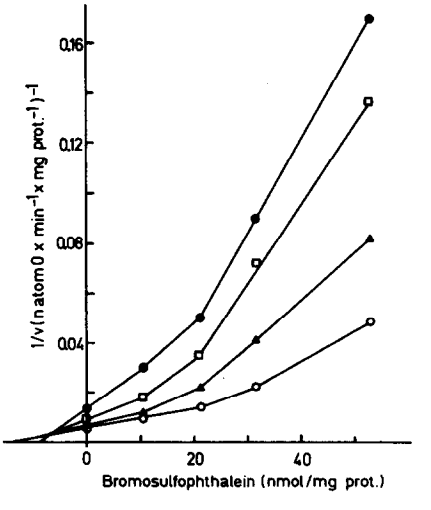

Fig. 5. Dixon plot of succinate respiration in the uncoupled state in the presence of bromosulfophthalein. Values of $1/v$ taken from Fig. 3 are plotted against the bromosulfophthalein concentration. The succinate concentrations were 0.45 mM (●), 0.9 mM (□), 1.8 mM (△), and 3.6 mM (○).

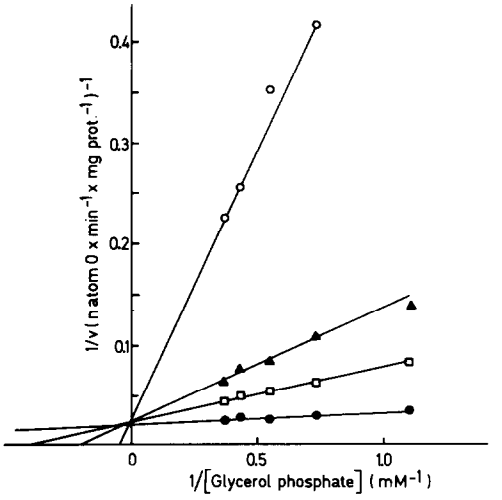

Fig. 6. Lineweaver–Burk plot of glycerol phosphate respiration in the uncoupled state at various bromosulfophthalein concentrations. Incubation conditions were as in Fig. 1 with the exception that ADP was omitted and 1 μM CCP added. The bromosulfophthalein concentrations in nmole/mg protein were: none = control (■), 12.5 (□), 25.0 (▲), and 50 (○).

more strongly inhibited by the dye. In the case of succinate, where at low bromosulfophthalein concentrations the electron flow via methylene blue to oxygen is slower than via the respiratory chain, methylene blue itself may affect the dehydrogenase (succinate (acceptor) oxidoreductase; EC 1.3.99.1) [8]. Alternatively, the intramitochondrial methylene blue concentration may not be sufficient, simulating a slow rate of dehydrogenase action. In all other cases, respiration and dehydrogenase reactions are strictly inhibited in parallel.

Respiration of sonic submitochondrial particles is also inhibited by bromosulfophthalein (Fig. 8). The NADH dehydrogenase (reduced-NAD: (acceptor) oxidoreductase; EC 1.6.99.3) which can be measured here

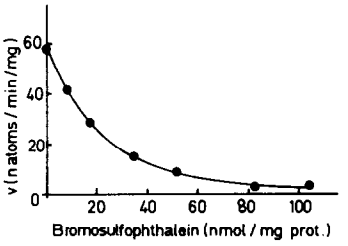

Fig. 8. Inhibition by bromosulfophthalein of the respiration of β-hydroxybutyrate of submitochondrial particles. Submitochondrial particles were suspended in 1 ml buffer consisting of 10 mM Tris, 15 mM K_2HPO_4, 3 mM $MgSO_4$, at pH 7.4; 0.88 mg protein/ml. Respiration was started by addition of 0.2 mM NAD^+ and 4 mM β-hydroxybutyrate.

directly by use of NADH as substrate, turned out to be very insensitive to bromosulfophthalein inhibition. As much as 150 nmole/mg protein were needed for half maximal inhibition. The various dehydrogenases seem to be inhibited at different concentrations of bromosulfophthalein. In Table 2, I_{50} values for oxygen consumption by respiration or shunted dehydrogenases under different conditions are listed. Oxidation of pyruvate is hardly inhibited by bromosulfophthalein. Dehydrogenase (D-3-hydroxybutyrate: NAD^+ oxidoreductase, EC 1.1.1.30) and respiration of β-hydroxybutyrate are inhibited with nearly identical I_{50} values. For succinate as substrate, the table further shows that in state 4, where dehydrogenases are not rate limiting, higher concentrations of bromosulfophthalein are required for half-maximal inhibition.

Difference spectra

Difference spectrophotometric measurements serve to identify a specific inhibitory site since they allow determination of a change in the relative amounts of oxidized and reduced respiratory chain components. Figure 9 shows the difference spectra of

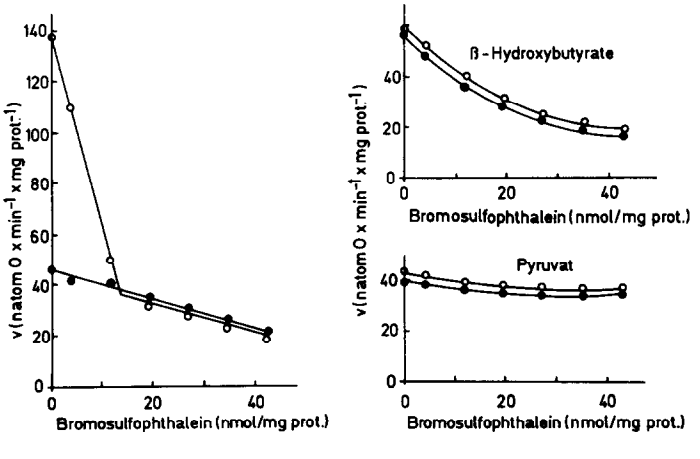

Fig. 7. Comparison of inhibition of uncoupled respiration and of dehydrogenases with different substrates. Mitochondria (3.2 mg protein/ml) were incubated in KCl medium (140 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, at pH 7.3). The concentrations of the respective substrates were 4 mM. Respiration (○) was measured after addition of 1 μM CCP. The dehydrogenases (●) were measured on addition of 3 μM rotenone, 2 μg antimycin A/ml and 1 mM methylene blue.

Table 2. Comparison of the inhibition by bromosulfophthalein of respiration and dehydrogenases

	Respiration		
	Controlled	Uncoupled	Dehydrogenase
Added substrate		I_{50} (nmole bromosulfophthalein/mg protein)	
Succinate 2 mM	35	10	27
4 mM	38	11	37
Pyruvate 2 mM		No significant inhibition up to 70	
4 mM		No significant inhibition up to 70	
β-Hydroxybutyrate 2 mM	20	18	16
4 mM	21	19	18
Glycerol phosphate 4 mM	Not measured	14	Not measured

Conditions were as in legend to Fig. 7.

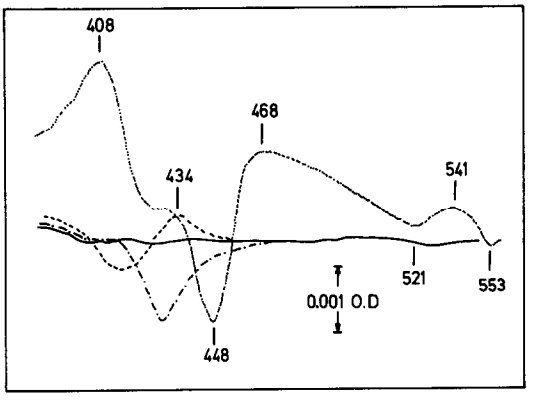

Fig. 9. Mitochondrial difference spectra on bromosulfophthalein addition. Difference spectra were taken by an Aminco–Chance spectrophotometer from two 10-mm cuvettes each containing 3 ml mitochondrial suspension (5 mg protein) in sucrose medium (250 mM sucrose, 5 mM Tris, 2 mM pyruvate, 0.5 mM malate), at ambient temperature and pH 7.2. Spectrum resulting immediately after addition of 20 nmole bromosulfophthalein/mg protein to one cuvette (– – –) and 5 min thereafter (—·—·—); spectrum oxidized by addition of 1 μM CCP against reduced by addition of 1 mM KCN (·····). Scanning speed 2 mm/sec; range of scale 0.005 absorption units.

mitochondrial suspensions without and with 20 nmole bromosulfophthalein/mg protein, taken immediately and 5 min after bromosulfophthalein addition.

Following bromosulfophthalein addition there is a minimum at 414 nm and a maximum at 434 nm which probably reflect increased oxidation of flavoproteins. Later a minimum at 429 nm arises suggesting an increasing amount of oxidized cytochrome *b*. Thus, addition of 20 nmole bromosulfophthalein/mg protein primarily results in oxidation of flavoproteins, followed by a slight oxidation of cytochrome *b*, whereas all other cytochromes remain unchanged. The quality of the spectral changes is independent from the substrate used. Thus, it is the electron transport to flavoproteins which is inhibited.

Effect of pH and temperature on respiratory inhibition by bromosulfophthalein

(*a*) *Effect of pH.* Inhibition of respiration by bromosulfophthalein is only slightly affected by variation of the pH of the mitochondrial suspension. On increase of pH from 6 to 9, inhibition is diminished by about 25 per cent. Under the same conditions, the respiratory control ratio decreases to a similar extent.

(*b*) *Effect of temperature.* The dependence of mitochondrial respiration on bromosulfophthalein concentration was measured at various temperatures. In Fig. 10, the respiratory rates of the substrate β-hydroxybutyrate at various temperatures are reciprocally plotted against the concentration of bromosulfophthalein. I_{50} values obtained from this plot showed that increasing temperature appears to diminish the inhibitory effect of bromosulfophthalein. This suggests that bromosulfophthalein might act by increasing the activation energy for respiration. But as can be seen from the Arrhenius plot (Fig. 11), above 20°, the activation energy, as calculated from the slope of the curve, is about 14 kcal/mole (58 kJ/mole) independent of the concentration of bromosulfophthalein. The calculated activation energy is in good agreement with the results obtained by others for dehydrogenase reactions [9, 10]. Similar Arrhenius plots were obtained for other substrates and also for state 3 respiration. In contrast to nonionic detergents which decrease the activation energies of dehydrogenase reac-

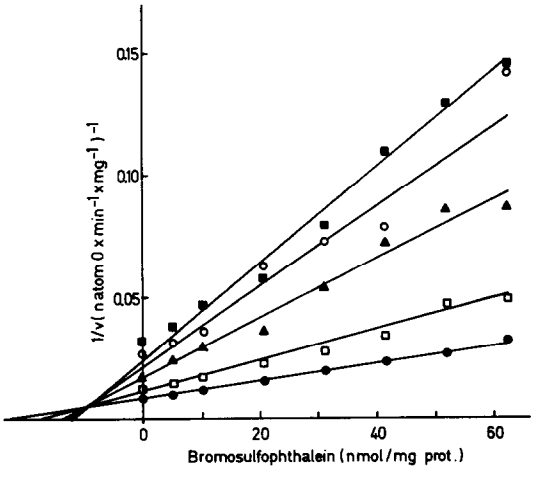

Fig. 10. Dependence on temperature of respiratory inhibition by bromosulfophthalein. Mitochondria (2.4 mg protein/ml) were incubated in KCl medium (140 mM KCl, 1 mM $MgSO_4$, 5 mM Tris, 1 mM K_2HPO_4, 4 mM β-hydroxy-butyrate; pH 7.2). Respiratory rates were measured on uncoupling by 1 μM CCP as a function of the bromosulfophthalein concentration at different temperatures: 15° (■), 20° (○), 25°(▲), 30° (□), and 35° (●).

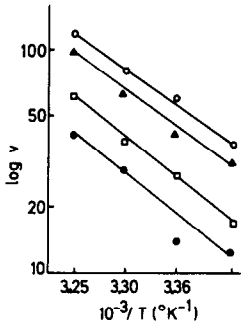

Fig. 11. Arrhenius plot of hydroxybutyrate respiration at various bromosulfophthalein concentrations. Respiratory rates after different bromosulfophthalein additions were taken from Fig. 10 and plotted against $1/T$. Bromosulfophthalein addition nmole/mg protein: none = control (○), 5.2 (▲), 20.8 (□), and 42 (●), respectively.

tions [11], bromosulfophthalein does not affect the activation energies of respiration.

DISCUSSION

Bromosulfophthalein action in mitochondrial respiration can be differentiated into two different effects.

(1) The most sensitive is state 3 respiration which is inhibited with a K_i of about 3 nmole/mg protein. The experiments are in agreement with the assumption that transport of inorganic phosphate is inhibited by bromosulfophthalein [2]. Recently, this has been proved to be true by more direct measurements [4]. Although there is some degree of competition between bromosulfophthalein and inorganic phosphate, there is no normal competitive Michaelis–Menten kinetics, as postulated by Laperche and Oudea [4]. The Dixon plot is nonlinear even if we use the data of these authors. (It is not clear how these authors arrived at the small K_i values reported in their Table 1. Using their data, we would calculate values of almost an order of magnitude greater. Such values would be consistent with ours.) The Dixon plot will be linear, however, when the reciprocal velocity is plotted against the square of the dye concentration. This may indicate that not one [4], but two molecules of bromosulfophthalein are bound at or near the phosphate carrier as inhibitive unity.

Similar Dixon plots with an upward bend have been described for inhibition of ATPase by acyl-CoA compounds [12] and for inhibition of phosphate translocation by mersalyl [13].

It is worthwhile emphasizing the similarity betwen mersalyl, a well-known inhibitor of phosphate translocation, and bromosulfophthalein action (Table 1). These two compounds are similar in their amphiphilic character. In addition, mersalyl is covalently attached to SH groups, whereas bromosulfophthalein is known to be conjugated in the liver with glutathion [14,15]. One important difference, however, is that bromosulfophthalein action is readily reversed by albumin addition, as shown in a following paper [16].

(2) At concentrations of 20–100 nmole/mg protein, respiration in state 4 or in uncoupled state is inhibited, too. Since not only transport of phosphate, but also that of taurocholate into isolated hepatocytes is strongly and competitively inhibited by bromosulfophthalein [17], it might be a general effect of the dye to inhibit transport of anions, as has been shown recently for other substrates [4]. Inhibitory effects on glycerol phosphate respiration and on respiration of submitochondrial particles, however, show that permeation is at least not the only process it interferes with. The experiments clearly showed that the dehydrogenases become inaccessible. The effect of an increased negative surface charge density [1] presumably is the ultimate parameter switching the accessibility of dehydrogenases for substrate anions. This is in agreement with the finding of Mehlhorn and Packer [18] on submitochondrial particles that respiratory inhibition imposed by ionic detergents is reversed by the addition of a detergent with opposite charge. Even soluble dehydrogenases, enclosed in phospholipid bilayer membranes, have been shown to be sensitive to a change in surface charge [19, 20].

Although Killenberg and Hoppel [2] did not observe this effect on dehydrogenase reactions, it can be derived from their data that at bromosulfophthalein concentrations above 5 μg/mg protein, inhibition is markedly dependent on the substrate used. This reflects, in our opinion, the varying sensibility of the different dehydrogenases.

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