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THE INHIBITION BY BROMOTHYMOL BLUE OF ANION TRANSLOCATION ACROSS THE MITOCHONDRIAL MEMBRANE

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SUMMARY

1. In rat liver mitochondria bromothymol blue inhibited the exchange of [^{14}C]succinate for succinate, malonate, L-malate and inorganic phosphate; the [^{14}C]citrate/citrate and [^{14}C]citrate/malate exchange reactions and the phosphate/hydroxyl exchange were also inhibited by this dye. The inhibition of the rate of succinate, citrate and phosphate uptake by bromothymol blue is found to be competitive.

2. The degree of inhibition by bromothymol blue of the [^{14}C]succinate/malonate exchange reaction was pH dependent. It has been shown that the inhibition increased linearly while the pH was increased from 6.0 to 8.2. However, the binding rate of bromothymol blue to the mitochondria decreased with the rising pH of the medium. It is concluded that the binding of acidic bromothymol blue was not essential for the inhibitory effect.

3. Other sulfonephthalein derivatives also inhibited [^{14}C]succinate/malonate exchange reaction. At pH 7.2 the relative order of the strength of the inhibitory action of the sulfonephthalein compounds tested was: thymol blue $>$ bromocresol green $>$ bromothymol blue $>$ phenol red $\gg$ bromocresol purple. The results do not indicate any correlation between the pK values of pH indicators and their extents of inhibition.

4. It is suggested that the negatively charged bromothymol blue interacts with the positively charged centers of the anion carrier systems causing inhibition of membrane permeability for anions.

INTRODUCTION

Bromothymol blue is known to be bound by various proteins. Antonini et al. [1] have found that the rate of association between bromothymol blue and hemoglobin is profoundly affected by O_2 pressure. The authors suggested that this effect is directly related to the allosteric interaction. Ullmann et al. [2] have shown that bromothymol blue binds more tightly to phosphorylase *a* than phosphorylase *b* and the binding of AMP to phosphorylase *b* increases the affinity of this protein for the dye. Kvame et

al. [3] reported that bromothymol blue stimulates the glutaminase activity in pig kidney and counteracts the inhibitory action of chloride. Bromothymol blue is known to be bound also by mitochondria and other membrane systems. Saris and Kuronen [4] have shown that structural proteins are responsible for the binding of the dye in mitochondria. Chance and coworkers [5, 6] used bromothymol blue to record inside pH changes of the mitochondrial suspension. It has also been shown that pH indicator (bromothymol blue) is bound to the membranes of submitochondrial particles, of bacterial chromatophores and of chloroplast grana and it has been concluded that it indicates the inside pH in these preparations [7, 8]. On the contrary, Cost and Frenkel [9], and Jackson and Crofts [10] reported that the absorbance change of the dye reflected a conformational change in the chromatophores. According to Mitchell et al. [11] the interpretation of the results obtained by bromothymol blue technique for measurement of internal pH is difficult because the dye may migrate, and it is not known whether the inner aqueous phase or the membrane phase, or both are affecting the dye. Colonna et al. [12] analyzed the properties of the bound bromothymol blue and the nature of the force driving its interaction with the mitochondrial membrane. They suggested that the absorbancy changes of bromothymol blue reflect changes in the activity coefficient rather than pH changes and thus bromothymol blue acts as a probe of electrostatic or hydrophobic interactions.

In a previous paper we have reported [13] that bromothymol blue inhibits succinate oxidation in intact rat liver mitochondria and suggested that the inhibition is caused by a direct action of bromothymol blue on succinate transport into mitochondria. Later we have shown [14] that bromothymol blue inhibits mitochondrial respiration also with other substrates. Hosoi and his associates [15] observed competition between P_i and bromothymol blue in photosynthetic ATP formation in chromatophores of *Rhodospirillum rubrum*. Killenberg and Hoppel [16] reported the inhibition of rat liver mitochondrial oxidative phosphorylation by one of the sulfonephthalein derivatives, bromosulfonephthalein. These authors suggest that bromosulfonephthalein inhibits oxidative phosphorylation by inhibiting P_i transport across mitochondrial membrane.

Using isotopic technique in the present paper we provide the evidence that bromothymol blue inhibits the transport of some metabolite anions through the mitochondrial membrane.

MATERIALS AND METHODS

Sulfonephthalein derivatives were obtained from Merck Co. Inc. Mersalyl, rotenone, antimycin A, oligomycin, succinate and citrate were purchased from Sigma Chem. Co.; L-malate, malonate from Koch-Light; carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) from Calbiochem; 1,2,3-benzenetricarboxylic acid from Merck-Schuchardt; [1,5-^{14}C]citrate from the Radiochemical Centre, Amersham; [1,4-^{14}C]-succinate from Isocommerz GMBH (GDR); [^{32}P]phosphate from I.B.J. Świerk (Poland). Butylmalonate was generous gift from Dr Popinigis.

To prepare mitochondria we adopted the procedure of Loewenstein et al. [17]. Rat liver (10g of tissue) was rinsed with ice-cold 0.25 M sucrose (Ca^{2+} free)–10 mM Tris–HCl (pH 7.4), dried between filter paper, thoroughly minced and washed, and homogenized manually in a Potter–Elvehjem homogenizer with a Teflon pestle. The

15 % homogenate was centrifuged for 1 min at 3200 × *g*, and the supernatant centrifuged for 2 min at 17 000 × *g*. The precipitate was suspended by homogenization in 12 ml 0.25 M sucrose–10 mM Tris–HCl (pH 7.4) and centrifuged for 4.5 min at 19 000 × *g*. The fluffy layer on the pellet was removed by washing and the pellet was resuspended in 5 ml 0.25 M sucrose–Tris and centrifuged for 5.5 min at 19 000 × *g*. The pellet was finally suspended in 1.5 ml 0.25 M sucrose. 0.25 M sucrose solution was deionized by passing through a mixed-bed ion-exchange resin (Amberlit MB-3, BDH). Protein was measured by ultraviolet absorption [18, 19]. Respiration was measured with a Clark oxygen electrode at 23 °C in 2.4 ml medium (pH 7.4) containing: 15 mM KCl, 50 mM Tris–HCl, 5 mM $MgSO_4$, 1 mM potassium phosphate, 4 μg rotenone, 1 μM CCCP and succinate (Tris salt) as indicated in the figures. The reaction was started by addition of 50 μl mitochondrial suspension (1.5 mg protein). Bromothymol blue was dissolved in ethanol and added in a volume of 2 μl. Further details are specified in the legends.

Methods used for determination of the [^{32}P] phosphate, [^{14}C]succinate and [^{14}C]citrate exchange reaction as well as for [^{14}C]succinate and [^{14}C]citrate loading were essentially according to the procedure described previously [20]. Particular details of each experiment will be found in the legends to figures and tables.

The measurement of the kinetics of the dicarboxylate transport was tested as uptake of [^{14}C]succinate [21] at 18 °C. The medium was made of 125 mM KCl, 20 mM Tris–HCl (pH 7.4), rotenone (1 μg/ml) and antimycin A (2 μg/ml). The reaction was initiated with [^{14}C]succinate and stopped after 15 s with 10 mM butylmalonate, followed immediately by centrifugation in a microcentrifuge. The pellet was dissolved in 0.5 ml of a 10 % $HClO_4$ and its radioactivity estimated by liquid scintillation counting. Correction was made for the sucrose-permeable space by subtracting values obtained when butylmalonate was added before succinate.

The kinetics of tricarboxylate transport was measured as uptake of [^{14}C]-citrate at 7 °C with mitochondria preloaded with citrate [22] in a medium the same as that used in experiments on succinate uptake. The exchange was initiated by addition of [^{14}C]citrate and stopped with 10 mM benzenetricarboxylate after 15 s. Determination of radioactivity in pellet and correction for sucrose permeable space were made as in succinate uptake experiments.

The kinetics phosphate transport through the phosphate–hydroxyl carrier was tested as uptake of [^{32}P]phosphate at 7 °C. The medium was made of 125 mM KCl, 20 mM Tris–HCl (pH 7.4), rotenone (2 μg/ml), antimycin A (0.5 μg/ml), oligomycin (3.3 μg/ml) and 8 mM butylmalonate. The reaction was initiated by addition of [^{32}P]phosphate and stopped after 15 s with 1 mM mersalyl followed by centrifugation. Extraction of pellet and correction for sucrose-permeable space was made as noted above. For the transport of succinate, citrate and phosphate the amount of mitochondria ranged between 2.5 and 3.2 mg and the final volume was 1 ml.

The H_2O of the osmotically-active space of mitochondria (incubated for 2 min at 7 °C in the same medium which used for anion transport measurements) in the absence or in the presence of bromothymol blue at the various concentrations was determined with [^{3}H]H_2O and [^{14}C]sucrose, according to the method described by Palmieri et al. [23].

For electron microscopy samples of mitochondria were fixed in glutaraldehyde,

then in OsO_4, embedded in epon, and sectioned. Specimens were examined as described previously. [24]

Bromothymol blue binding to rat liver mitochondria was determined according to Chance [5] in samples parallel to the sample in which the effect of bromothymol blue on anion transport was studied. After 2 min of incubation mitochondria were centrifuged at 15 000 $\times g$ for 1.5 min. The pellet was washed and then clarified by the addition of Triton X-100 and made alkaline to pH 9. Optical measurements of the bromothymol blue absorption were made at 620 nm on a Unicam SP-800 spectrophotometer.

RESULTS

Effect of bromothymol blue on mitochondrial respiration with succinate as substrate

The effect of bromothymol blue on succinate oxidation in the uncoupled state is shown in Fig. 1. The addition of bromothymol blue (10 μM) to a suspension of liver mitochondria oxidizing succinate (2 mM), in the presence of CCCP caused immediate inhibition of respiration. This inhibition was reversed by 5 mM succinate. The inhibi-

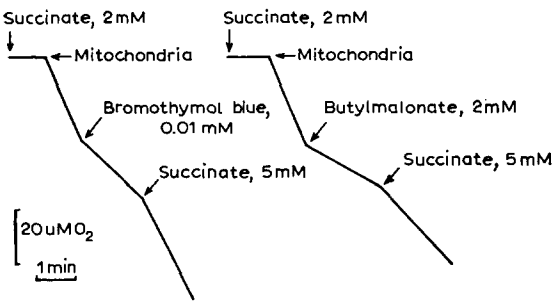

Fig. 1. Effect of bromothymol blue and butylmalonate on succinate oxidation in intact rat liver mitochondria. Experimental conditions were as described under Materials and Methods.

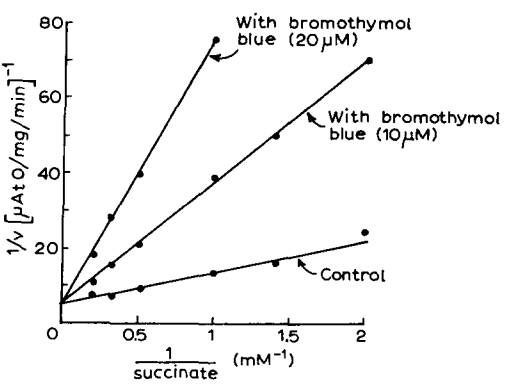

Fig. 2. Double reciprocal plots of the rate of succinate oxidation against succinate concentration in the presence of various concentrations of bromothymol blue as indicated. Experimental conditions were as described under Materials and Methods.

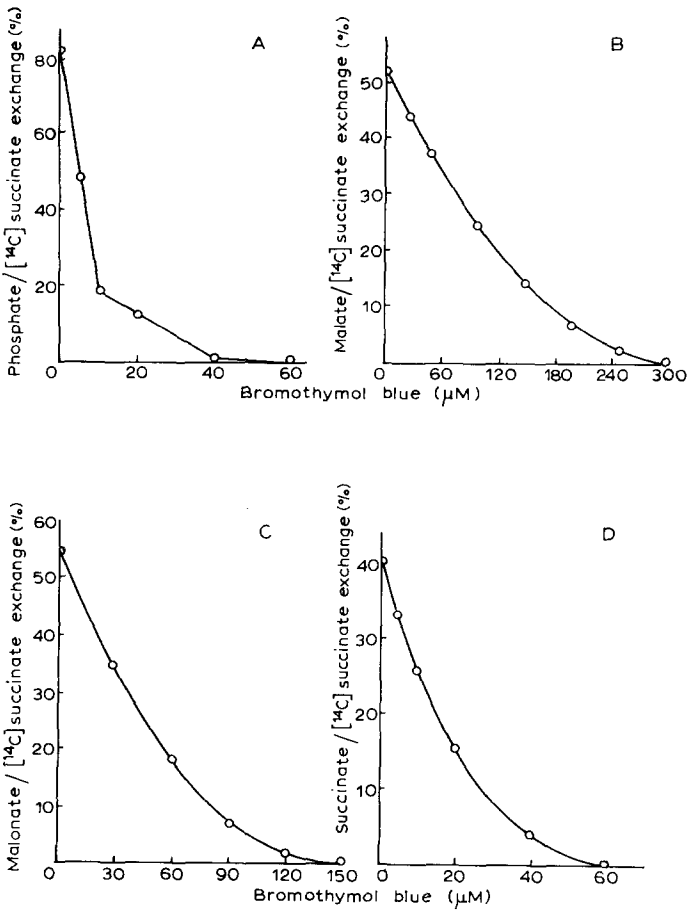

Fig. 3. Effect of bromothymol blue on phosphate/[^{14}C]succinate (A), L-malate/[^{14}C]succinate (B), malonate/[^{14}C]succinate (C), succinate/[^{14}C]succinate (D) exchange reactions. Rat liver mitochondria (4.4 mg of protein) prelabelled with [^{14}C]succinate according to Robinson and Williams [25] were added to 1 ml of incubation medium containing: 125 mM KCl, and 20 mM Tris–HCl (pH 7.25). 1 mM phosphate, L-malate, malonate or succinate were added to corresponding experiments. Bromothymol blue was added in ethanol solution at concentration indicated in the figure (maximal volume of bromothymol blue solution was 2 μl). 2 μl of ethanol was added to control sample. After 2 min incubation at 5 °C except in experiment (A) where temperature was 10 °C, 25 mM butylmalonate was added and the samples were immediately centrifuged at 14 000 $\times g$ for 1.5 min. Then 0.8 ml of supernatant were drawn off and added to 0.5 ml of 10 % $HClO_4$. The acidified supernatants were centrifuged at 3000 $\times g$ for 5 min. Mitochondrial pellets were resuspended in 0.5 ml 10 % $HClO_4$, extracted for 30 min and then centrifuged at 14 000 $\times g$ for 3 min. 0.1 ml of supernatant and pellet extracts were added to a glass counting vial. The radioactivity was determined in a spectrometer for liquid scintillation Isocap 300 after 10 ml of scintillator solution (which contained 4 g PPO and 0.2 g POPOP in 0.7 l toluene + 0.3 l methanol) had been added. The percentage exchange equals: supernatant dpm after incubation with anion − supernatant dpm after incubation without anion/mitochondrial dpm after incubation without anion × 100.

tory effect of bromothymol blue on succinate oxidation in uncoupled state was compared with the effect of a known inhibitor of the dicarboxylic acid transporter butylmalonate [25]. Data presented in Fig. 1 show that 10 μM bromothymol blue

inhibited succinate oxidation in a similar way to 2 mM butylmalonate.

Bromothymol blue, even at relatively high concentration, was without effect on succinate oxidation in broken mitochondria or submitochondrial particles [13]. Inhibitory effect of bromothymol blue on succinate oxidation in intact rat liver mitochondria in metabolic state 3 and in the ion-pumping state has already been reported [13].

In Fig. 2 a Lineweaver and Burk plot is presented showing apparent competition between succinate and bromothymol blue.

Inhibition of dicarboxylic transport by bromothymol blue

The effect of bromothymol blue on dicarboxylate carrier was studied using succinate, malonate, L-malate and phoshate as substrate. Rat liver mitochondria loaded with [^{14}C]succinate were added to the medium containing 1 mM anion. In the controls (those without the addition of anion) a significant efflux of [^{14}C]-succinate occurred. Bromothymol blue had practically no effect on this efflux (not shown). The presence of the anion in the incubation medium caused a rapid and extensive efflux of labelled succinate from mitochondria. The exchange reaction was inhibited by the bromothymol blue if it was present in the incubation medium. The exchange reactions of the dicarboxylic acid transporting system seem in general to be sensitive to the bromothymol blue, but the degree of inhibition depended on the anion exchanged (Figs 3, 4). The [^{14}C]succinate/P_i exchange was completely inhibited by 40 μM bromothymol blue, the [^{14}C]succinate/succinate exchange required 60 μM bromothymol blue for complete inhibition. Less sensitive to bromothymol blue were [^{14}C]succinate/malonate and [^{14}C]succinate/malate exchange reactions. For a complete inhibition of the [^{14}C]succinate/malonate exchange, 150 μM bromothymol blue was required, and [^{14}C]succinate/

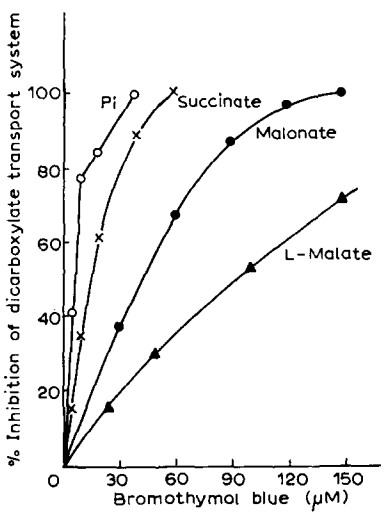

Fig. 4. Percentage of inhibition of anion/[^{14}C]succinate exchange reaction by different concentrations of bromothymol blue. Experiments were performed as described in Fig. 3. The percentage inhibition of the dicarboxylate carrier is calculated by taking the percentage exchange after 2 min in the absence of bromothymol blue as the baseline value.

malate exchange was inhibited completely only by 300 μM bromothymol blue.

Since the concentrations of pH indicator used in these experiments are rather high, suspicion existed that bromothymol blue might cause damage of the mitochondrion. Control of this aspect was provided by the electron microscopie observations and by measurements of volume in the inner mitochondrial compartment. Electron microscopie observations of mitochondria incubated in samples, parallel to the samples in which the effect of bromothymol blue on anion translocation was examined, did not show damage of mitochondrial structure. In the volume measurements (see Materials and Methods) it was observed that the sucrose-inaccessible water remained unchanged in the presence of bromothymol blue at the concentration range 20–200 μM (not shown).

It has been reported [26] that the sensitivity of dicarboxylate/dicarboxylate anion exchanges in rat liver mitochondria to 2-butylmalonate is depending on temperature. We compared the effect of bromothymol blue on [^{14}C]succinate/malonate exchange reaction at 5 °C and 20 °C. It may be seen from Fig. 5 that the percentage of inhibition by this dye at both temperatures was identical.

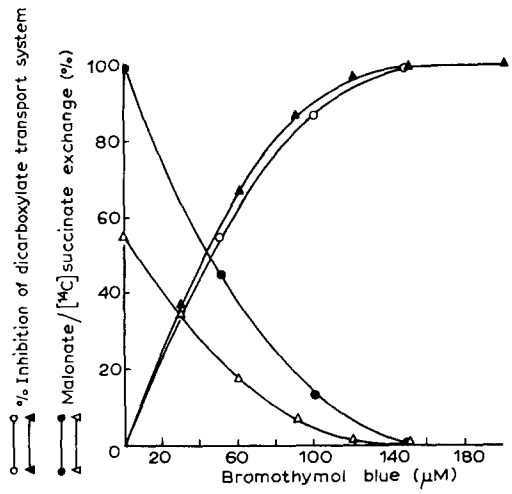

Fig. 5. The effect of bromothymol blue on malonate/[^{14}C]succinate exchange at 5 °C (△ — △) and 20 °C (● — ●). Experiments were performed as described in Fig. 3.

pH Dependence of the inhibition of [^{14}C]succinate/malonate exchange reaction by bromothymol blue

Recently Meisner [27] proposed that inhibition of metabolite anion uptake in mitochondria by tetraphenylboron depends on the negative charge of the boron anion. We assume that the mechanism of bromothymol blue action on the anion transport through the mitochondrial membrane, might be similar to that of tetraphenylboron; in that case, the percentage of inhibition has to be dependent on the amount of the anionic form of the dye. Taking into account the pK (7.1) of bromothymol blue [28], its effect on anion transport across the mitochondrial membrane at different pH's of the incubation medium was examined. Fig. 6 shows that [^{14}C]-

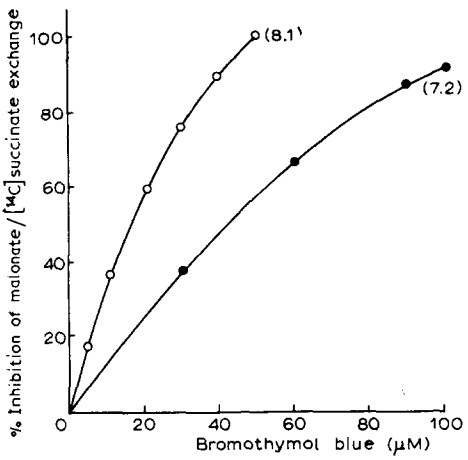

Fig. 6. Effect of bromothymol blue on malonate/[^{14}C]succinate exchange reaction at pH 7.1 (● — ●) and 8.1 (○ — ○). Experimental conditions were as described in Fig. 3.

succinate/malonate exchange reaction was completely inhibited by 50 μM bromothymol blue at pH 8.1, while at pH 7.2 50 μM bromothymol blue inhibited this exchange in about 55 %. In fig. 7 the effect of bromothymol blue on [^{14}C]succinate/malonate exchange reaction at pH of the medium from 6.0–8.2 is compared with the ionization curve of the dye calculated theoretically. It can be seen that raising the pH from 6.0 to 8.2 caused a linear increase of the inhibition of [^{14}C]succinate/malonate exchange reaction and an increase of the amount of the anionic form of the dye.

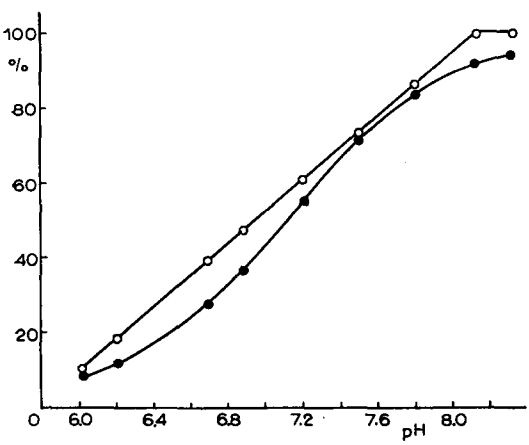

Fig. 7. pH dependence of the inhibition of malonate/[^{14}C]succinate exchange reaction by bromothymol blue. The percentage of the dye in ionized form at different pH values was calculated and is shown on the graph for comparison. Bromothymol blue was used at concentration 50 μM. Other experimental conditions as described in Fig. 3. ● — ●, ionisation of bromothymol blue; ○ — ○, inhibition of malonate/[^{14}C]succinate exchange.

Bromothymol blue binding to mitochondria at different pH of the medium

In an attempt to determine whether the increasing inhibitory effect of bromothymol blue at higher pH is parallel to an increasing binding of the dye to mitochondria, the effect of pH on bromothymol blue binding to mitochondria was tested. The results are shown in Fig. 8. It may be seen that with the increasing pH of the medium, bromothymol blue binding to mitochondria was decreasing, in agreement with previous results of Colonna et al. [12]. Thus it seems likely that the binding of the acidic form of bromothymol blue was not essential for the inhibitory effect.

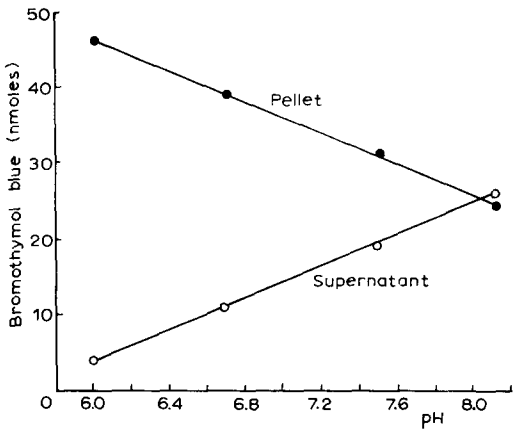

Fig. 8. The effect of pH on bromothymol blue binding to rat liver mitochondria. Experiments were performed as described under Materials and Methods.

Inhibition of [^{14}C]succinate/malonate exchange by some sulfonephthalein derivatives

Bradford and McGivan [29] recently reported that one of the sulfonephthalein derivatives, bromocresol purple, inhibits glutamate transport into rat liver mitochondria. The fact that bromothymol blue is an inhibitor of dicarboxylate carrier and that bromocresol purple is an inhibitor of glutamate transport across mitochondrial membrane, suggested that other sulfonephthalein derivatives might also inhibit permeability of mitochondrial membrane for anions. The effects of several sulfonephthalein compounds on the [^{14}C]succinate/malonate exchange reaction at the pH of the medium 7.2 or 8.3 are shown in Table I. This exchange reaction seems to be inhibited by every examined sulfonephthalein derivative at a concentration of 50 μM. However, differences in the degree of the sensitivity, depending on the sulfonephthalein compound used, were observed. The relative order of the strength of the inhibitory action of the sulfonephthalein derivatives tested was: thymol blue $>$ bromocresol green $>$ bromothymol blue $>$ phenol red $\gg$ bromocresol purple at pH 7.2; while at pH 8.3 it was: bromocresol green $>$ bromothymol blue $>$ thymol blue $>$ phenol red $>$ bromocresol purple. The results do not indicate any correlation between the pK values of the pH indicators and their extents of inhibition.

TABLE I

INHIBITION OF [^{14}C]SUCCINATE/MALONATE EXCHANGE BY SOME SULFONEPHTHALEIN DERIVATIVES

Sulfonephthalein derivatives were dissolved in ethanol and added to the incubation medium of volume 2 μl. Other experimental conditions were as described in Fig. 3, except the temperature was 9 °C.

Additions	pK (free indicator)	Experiment 1, pH 7.2		Experiment 2, pH 8.3	
		Exchange (%)	Inhibition (%)	Exchange (%)	Inhibition (%)
1 mM Malonate		68	—	77	—
+ 50 μM Bromocresol green[a]	4.7[b]	25	62	− 15[c]	122[d]
+ 50 μM Bromocresol purple	6.2	52	23	40	49
+ 50 μM Bromothymol blue	7.1	34	49	− 7[c]	109[d]
+ 50 μM Phenol red	7.8	39	43	33	57
+ 50 μM Thymol blue	8.9	19	72	22	73

[a] It was found that the tested sulfonephthalein derivatives did not interfere with the counting.

[b] According to Clark [28].

[c] Where a negative value is indicated, it means that in the presence of that pH indicator, the [^{14}C]succinate counts appearing in the supernatant are lower than in controls incubated in buffer only.

[d] A value greater than 100 % indicates that after incubation, the sample showed less extra-mito chondrial [^{14}C]succinate than controls with no added exchanging anion.

Inhibition by bromothymol blue of the exchanges of citrate and malate with [^{14}C]-citrate in rat liver mitochondria

The effect of increasing concentration of bromothymol blue on [^{14}C]citrate/

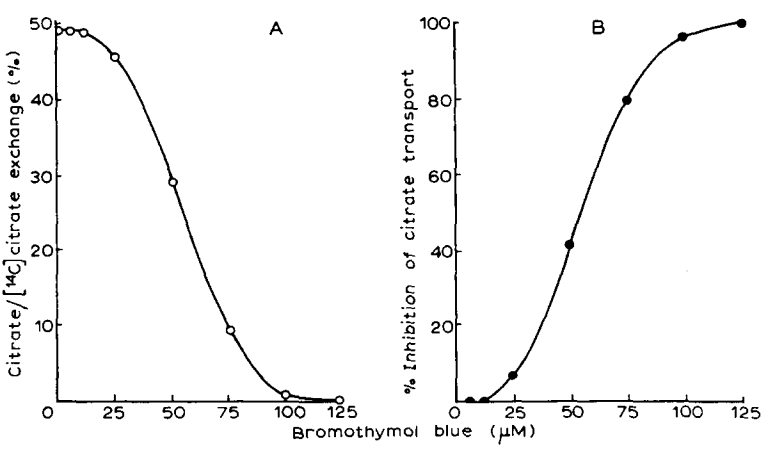

Fig. 9. Effect of bromothymol blue on citrate/[^{14}C]citrate exchange. Rat liver mitochondria (4.5 mg of protein) loaded with [^{14}C]citrate according to Halperin et al. [22] were incubated as described in Fig. 3 in media containing 1 mM citrate and bromothymol blue at the concentration as indicated in the figure. After 2 min incubation at 6 °C, 25 mM benzenetricarboxylate was added. Further procedures were as described in Fig. 3.

citrate and [^{14}C]citrate/malate exchanges is shown in Fig. 9 and Fig. 10. A 50 % inhibition of the exchange of 1 mM citrate with [^{14}C]citrate is brought about by 50 μM bromothymol blue. The inhibition by bromothymol blue of the exchange of 1 mM malate with [^{14}C]citrate was considerably weaker than the inhibition of the [^{14}C] citrate/citrate exchange. To achieve a 50 % inhibition of [^{14}C]citrate/malate exchange reaction, about 150 μM concentration of the dye was required. This indicates that the citrate/malate exchange reaction is about three times less sensitive to bromothymol blue than citrate/citrate exchange.

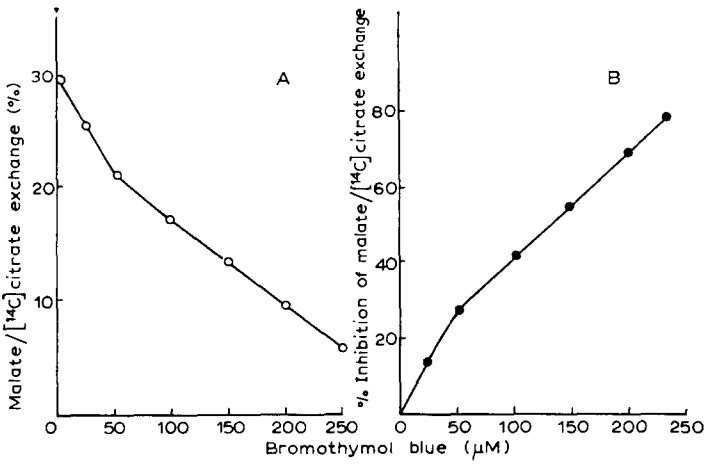

Fig. 10. Effect of bromothymol blue on malate/[^{14}C]citrate exchange. Experiments were performed as described in Fig. 9, except that 1 mM citrate was substituted by 1 mM L-malate.

Inhibition of [^{32}P]phosphate–hydroxyl exchange by bromothymol blue

Killenberg and Hoppel [16] reported the inhibition of rat liver mitochondrial oxidative phosphorylation by sulfobromophthalein. These authors suggest that bromosulfonephthalein inhibits oxidative phosphorylation by inhibiting P_i transport

TABLE II

INHIBITION OF [^{32}P]PHOSPHATE-UPTAKE BY BROMOTHYMOL BLUE

Mitochondria (3.6 mg protein) were preincubated 1 min at 5 °C in 1 ml of medium containing: 125 mM KCl, 20 mM Tris–HCl (pH 7.4), 2 μg rotenone, 0.5 μg antimycin A, 2 μg oligomycin, 5 mM butylmalonate and bromothymol blue where indicated. The reaction was initiated with 1 mM [^{32}P]phosphate (140 000 cpm), and stopped after 10 s with 1 mM mersalyl. The samples were immediately centrifuged. The pellets were suspended in 0.5 ml 10 % $HClO_4$, extracted for 30 min, and centrifuged at 14 000 × g for 3 min. Further procedure was as described in Fig. 3. Controls were incubated with 1 mM mersalyl added before the labelled P_i had been added, and the amount of radioactivity taken up was subtracted from the experimental samples, in order to arrive at the P_i incorporated into the matrix space.

Additions (μM)	Phosphate in matrix space (nmoles P_i/mg per min)	Inhibition (%)
None	27.5	—
50 bromothymol blue	15.7	43
100 bromothymol blue	5	82

across the mitochondrial membrane. Table II reveals that the phosphate transport through the phosphate–hydroxyl carrier was found to be very sensitive to bromothymol blue, being about 43 % and 82 % inhibited by 50 μM and 100 μM dye, respectively. These results strongly support suggestions of Killenberg and Hoppel [16].

Competitive inhibition of the dicarboxylate, tricarboxylate and phosphate carrier by bromothymol blue

The nature of inhibition of the rate of anion uptake by bromothymol blue was studied by varying the anion concentration at less than fully inhibiting amounts of bromothymol blue. As shown by the Lineweaver and Burk plots of Figs 11A, B and C, the addition of bromothymol blue to [^{14}C]succinate, [^{14}C]citrate or [^{32}P]-phosphate increases the K_m without changing the V of succinate, citrate and phosphate uptake, in agreement with a competitive type of inhibition. Under the assay conditions used (see Materials and Methods), external [^{14}C]succinate exchanges preferentially for internal phosphate [21]; external [^{14}C]citrate exchanges for internal citrate; and the uptake of phosphate proceeds through the P_i/OH^- carrier [30].

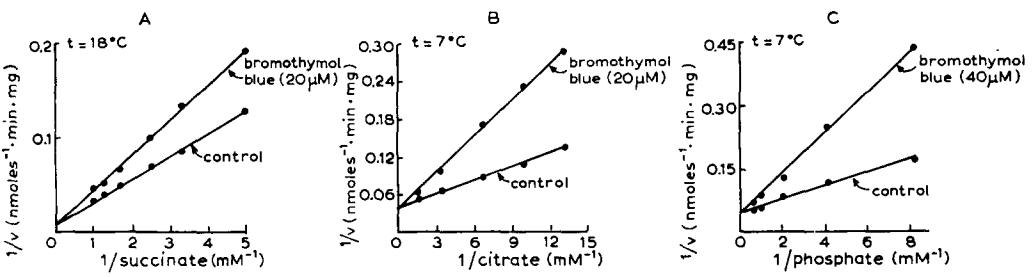

Fig. 11. Competitive inhibition by bromothymol blue of mitochondrial transport of succinate, citrate and phosphate. Conditions as described under Materials and Methods.

TABLE III

K_i VALUES OF MITOCHONDRIAL TRANSPORT SYSTEMS FOR BROMOTHYMOL BLUE

The K_i values were calculated from double reciprocal plots of the rate of anion uptake versus substrate concentration. Experimental conditions as described under Materials and Methods.

Transport system	K_i (M)
Succinate	$9.8 \cdot 10^{-6}$
Citrate	$3.0 \cdot 10^{-5}$
Phosphate	$7.1 \cdot 10^{-5}$

The K_i values of the above transport systems for bromothymol blue differ markedly (Table III). The least sensitive carrier is the phosphate–hydroxyl carrier (K_i = 71 μM) which is 2–7 times less sensitive to bromothymol blue than the tri- and dicarboxylate carriers (K_i = 30 μM and 9.8 μM, respectively). The competitive nature of inhibition of the dicarboxylate transport system by bromothymol blue is in agreement with the earlier conclusions down from studies on succinate oxidation (see Fig. 2).

DISCUSSION

The results presented in this paper clearly indicate that dicarboxylate, tricarboxylate and phosphate transporting systems in rat liver mitochondria are inhibited by bromothymol blue in a competitive manner with a K_i of 9.8 μM, 30 μM and 71 μM of dicarboxylate, tricarboxylate and phosphate carrier, respectively.

It has been reported [21, 31–34] that the mitochondrial membrane becomes less permeable to anions at alkaline pH. This change in membrane permeability behaviour led Ghosh and Chance [34] to the conclusion that the binding site for the anions on mitochondrial membrane is positively charged. It has been also reported that some cations stimulated either adenine nucleotides or other anions to be taken up by mitochondria [35]. This stimulation would be brought about by increasing the positive charge of the inner membrane resulting in a decrease of the apparent K_m for anions.

It is possible that negatively charged molecules of bromothymol blue combine, due to an electrostatic interaction, with the positively charged site of translocators resulting in a decrease of the binding anions, as only the positively charged site can bind anions. The consequence of it is an increase of the apparent K_m for anions with no change in V. This suggestion is in accordance with Klingenberg's et al. [36] observation that only the carrier–H^+ form can bind phosphate. This idea is strongly supported by the data presented on fig. 7. It can be seen that the degree of inhibition of anion translocation across mitochondrial membrane by bromothymol blue is markedly dependent on pH of the medium because both the amount of the anionic form of the dye and the inhibition increased with an increase of pH. The previously described [33, 37] inhibitory effect of the uncoupler on exchange-diffusion reactions of substrate anions can be explained in a similar way. The possibility of interaction between positively-charged sites of translocators and negatively-charged groups of anions, explain the inhibition of the dicarboxylate carrier by 2-oxoglutarate or citrate [21] and the reverse inhibition of the tricarboxylate carrier by some dicarboxylic acids [32].

On the other hand, as the chemical formulae of bromothymol blue and metabolite anion tested are quite different, observed competitive type of inhibition does not exclude the possibility that bromothymol blue and substrate anions attach to different sites and that the dye exerts its inhibition by an "allosteric" effect, as postulated by Winkler and Lehninger [38] for atractiloside inhibition of adenine nucleotide translocator.

It is worth mentioning that bromothymol blue at relatively low concentrations with pH above 7.0 rapidly and completely inhibits anions translocation across the mitochondrial membrane and therefore this dye may be used as an inhibitor in the "inhibitor stop method".

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