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Inhibition of Rat Liver Mitochondrial Oxidative Phosphorylation by Sulfobromophthalein

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SUMMARY

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The observation by others of decreased bile flow and death following sulfobromophthalein (BSP) infusion in rats and other laboratory animals suggested that BSP was a possible metabolic toxin and led to studies of its effect on mitochondria respiration. Measurements of oxygen consumption showed that state 3 oxidation of pyruvate, glutamate, succinate, β-hydroxybutyrate, palmitoyl-L-carnitine, and hexanoate by rat liver mitochondria was inhibited at 3 μg of BSP per milligram of mitochondrial protein, inhibition increasing with increased BSP concentration up to 24 μg/mg. Rat heart mitochondrial oxidation of pyruvate was similarly inhibited by BSP. BSP also inhibited incorporation of ^{32}P into ATP during state 3 respiration. Analysis of polarograph tracings indicated that BSP inhibited state 3 respiration without any consistent effect on state 4 or the ADP to oxygen ratio. Synthetic BSP-GSH conjugate did not inhibit mitochondrial oxygen consumption or prevent inhibition by unconjugated BSP. Adding defatted bovine albumin to the incubation medium decreased BSP inhibition of mitochondrial oxygen consumption. In addition, the introduction of albumin to the medium following inhibition by BSP caused a return to normal rates of oxygen consumption. The mechanism of the reversible inhibition of state 3 oxidation was investigated by studying the effect of BSP on some of the isolated steps of oxidative phosphorylation. BSP did not inhibit the succinate, glutamate, or β-hydroxybutyrate dehydrogenases, or the succinate–cytochrome *c* reductase or NADH oxidase reactions. Succinate oxidase was inhibited in whole mitochondria but not in sonicated submitochondrial particles. This suggested that BSP may act at the mitochondrial inner membrane, possibly by interfering with mitochondrial uptake of the substrates necessary for coupled oxidative phosphorylation. The effect of BSP was therefore measured at various concentrations of the substrates of coupled oxidative phosphorylation. There was no change in inhibition over a 50-fold range of ADP concentration. However, BSP inhibition varied inversely with concentration of P_i in the medium. Similarly, inhibition by BSP varied inversely with the concentration of succinate but was not affected by changing the concentration of the other oxidizable substrates. Since the mitochondrial uptake of succinate has been shown to require P_i, the data are consistent with an effect of BSP on P_i transport across the mitochondrial inner membrane. These studies suggest that BSP is a potent inhibitor of oxidative phosphorylation *in vitro*. There is no evidence at present, however, that BSP exerts a similar effect *in vivo*.

INTRODUCTION

Sulfobromophthalein is widely used in the clinical and experimental evaluation of hepatic function in man and laboratory animals. Following its administration to normal subjects, BSP[3] is rapidly concentrated in the liver, where it is conjugated predominantly to glutathione by the formation of a thioether bond between the phthalein ring and the glutathione sulfhydryl (1). While the thioether conjugate is readily secreted into the biliary tract, only a fraction of unconjugated BSP appears in bile, indicating that conjugation is prerequisite to efficient removal of BSP from the hepatocyte (2).

Toxic reactions to BSP have been observed infrequently following the usual diagnostic dose (5 mg/kg of body weight) in man. In these instances toxicity has usually been manifested as a skin rash or anaphylaxis, the latter occurring within 5 min of intravenous injection of BSP (3). In two reported fatalities the onset of clinical illness was delayed for up to 30 min after BSP administration, but no mode of toxicity was documented (4, 5).

Much larger doses of BSP have been fatal in laboratory animals. Recently two groups have observed that rats with biliary fistula died at varying time intervals after intravenous infusions of BSP (100–265 mg/kg/min). The cause of death was unexplained and was usually preceded by a decrease in the rate of bile flow (6, 7). Death has also been reported several hours after a single intravenous injection of 50–100 mg/kg into intact rabbits and dogs (8).

These observations in animals raised the possibility that BSP is a potential metabolic toxin and led to the present study of its effect on mitochondrial respiration. This report demonstrates for the first time that BSP is an effective inhibitor of oxidative phosphorylation in rat liver mitochondria. Evidence is offered that BSP may effectively block the transport of inorganic phosphate across the mitochondrial inner membrane.

METHODS

Preparation of mitochondria. Rat liver mitochondria were isolated from livers of male Wistar rats (175–250 g) according to Hoppel and Tomec (9). The livers were homogenized in 3 volumes of 300 mM sucrose, 5 mM morpholinopropanesulfonic acid, and 2 mM EDTA, pH 7.2, in a Potter-Elvehjem homogenizer with a loosely fitting Teflon pestle. Rat heart mitochondria and sonic submitochondrial particles were prepared as previously described (10, 11).

Oxidative phosphorylation. Oxygen consumption was measured in an incubation medium of 80 mM KCl, 50 mM morpholinopropanesulfonic acid, 1 mM EGTA, and, unless otherwise stated, 5 mM P_i. The final pH was 7.0; temperature, 30°; volume, 1.0 ml. Two milligrams of mitochondrial protein were added in each incubation. The rate of oxygen consumption was monitored with an oxygraph apparatus. Additions to the medium were made through a small aperture with micropipettes. Phosphate uptake was measured by incubating freshly prepared mitochondria in the above incubation medium, which also contained 10^6 cpm of $^{32}P_i$. Phosphorylation was estimated according to Hoppel and Cooper (12).

Specific activity was measured as nanoatoms of oxygen consumed per minute per milligram of mitochondrial protein in the oxidation experiments and, in the phosphorylation experiment, as the percentage of counts per minute of $^{32}P_i$ incorporated into organic phosphate multiplied by the concentration of phosphate in the medium

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[3] The abbreviations used are: BSP, sulfobromophthalein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

and expressed as nanomoles of P_i per minute per milligram of mitochondrial protein. Relative activity was calculated as specific activity in the presence of inhibitor, divided by control specific activity, then multiplied by 100 (i.e., as a percentage of control). State 3 and state 4 respiration and the ADP to oxygen ratio were measured according to Chance and Williams (13, 14).

Enzyme assays. Succinate dehydrogenase was assayed as described by Hoppel and Cooper (15). Succinate–cytochrome *c* reductase was measured by following the reduction of oxidized cytochrome *c*, as previously described (16), with the exception that 50 mM morpholinopropanesulfonic acid, pH 7.5, was used in place of phosphate buffer. Glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase were assayed spectrophotometrically by following the reduction of NAD at 30° as previously described (15). NADH oxidase and succinate oxidase were measured by the method of Blair, Oda, Green, and Fernandez-Moran (17).

The details of other methods are noted in the legends to the figures and tables. Protein was estimated by the biuret reaction following solubilization with deoxycholate (18).

Materials. Crystalline sulfobromophthalein was obtained through the courtesy of the Hynson, Wescott, and Dunning, Baltimore, or was purchased as a 50 mg/ml solution of sulfobromophthalein sodium, USP, from the Vitarine Company, New York. The latter preparation contained 2.5 μg/ml of EDTA as a preservative. Equimolar amounts of either preparation of BSP could be used interchangeably without any difference in effect.

The glutathione thioether conjugate of BSP was synthesized according to Whelan, Hoch, and Combes (2) and purified by chromatography over Dowex 50 (19). In an alkaline medium the optical density per milligram of synthetic BSP-GSH at 575

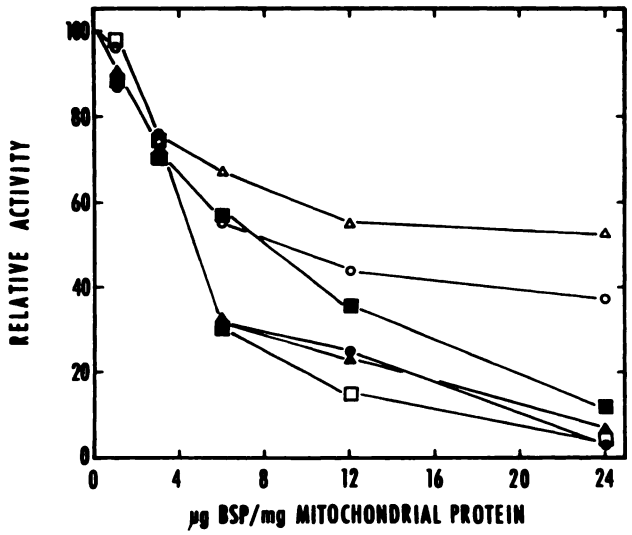

FIG. 1. *Inhibition of state 3 hepatic mitochondrial oxygen consumption by BSP*

Each experiment was started by addition of 40 μl containing 2 mg of mitochondrial protein to 1 ml of incubation medium. Endogenous substrates were depleted by two sequential additions of 10 μl of 13 mM ADP. Substrate in 10–25 μl was added, followed by 10 μl containing the indicated amount of BSP in aqueous solution. State 3 respiration was then initiated by addition of 50 μl of 100 mM ADP. The specific activity of state 3 respiration was calculated from the slope of the oxygraph tracing between 15 and 60 sec after the last addition of ADP. Substrate concentrations and control specific activities (without BSP) were as follows: △, 10 mM hexanoate + 0.5 mM L-malate, 58.6; ○, 20 mM DL-β-hydroxybutyrate, 59.7; ■, 10 mM pyruvate + 2.5 mM L-malate, 71.1; ▲, 0.02 mM palmitoyl-L-carnitine + 0.5 mM L-malate, 96.5; □, 10 mM glutamate, 109; ●, 10 mM succinate + 0.004 mM rotenone, 175.

nM was 81% of that of crystalline BSP, compared with a predicted 78% (2). Thin-layer chromatography of the product on Eastman silica gel plates developed in acetone–glacial acetic acid–butanol–water (6:1:1:3 by volume) resulted in a single spot which became purple on exposure to concentrated ammonia vapor and was ninhydrin-positive. In this system the product migrated with an R_f of 0.75 and could be distinguished from unconjugated BSP (R_f 0.87) and glutathione (R_f 0.61).

Defatted bovine serum albumin was prepared according to the method of Chen (20) and dialyzed as outlined by Hanson and Ballard (21). Palmitoyl-L-carnitine was synthesized according to Bremer (22).

GSH, ADP, and ATP were obtained from P-L Biochemicals. $^{32}P_i$ was purchased from International Chemical and Nuclear Corporation, Irvine, Cal. Other chemicals were of reagent quality and were obtained commercially.

RESULTS

Inhibition of mitochondrial oxidative phosphorylation. Substrate-initiated, ADP-dependent oxygen consumption (state 3 respiration) by freshly prepared rat liver mitochondria was studied using six different substrates (Fig. 1). The addition of BSP to the medium inhibited oxidation of each of the substrates, inhibition increasing with increased concentrations of BSP in each case. In other experiments the addition of BSP during state 3 respiration resulted in the same degree of inhibition as when BSP was added before the addition of substrate and ADP.

Oxidation was inhibited 25% with all substrates at 3 μg of BSP (3 nmoles) per milligram of mitochondrial protein. At higher concentrations of BSP the degree of inhibition varied with the substrate, and at 24 μg/mg it ranged from 52% with hexanoate plus L-malate to over 90% with succinate plus rotenone, glutamate, and palmitoyl-L-carnitine plus L-malate. At this higher concentration the extent of inhibition by BSP was greatest with those substrates having the highest state 3 specific activity prior to inhibition.

Concentrations of BSP above 36 μg/mg occasionally caused lysis of mitochondria, as noted by clarification of the suspension and cessation of all respiration. No lysis was seen below 30 μg/mg, where in all cases respiration proceeded at a constant rate for more than 1 min even at 90% inhibition.

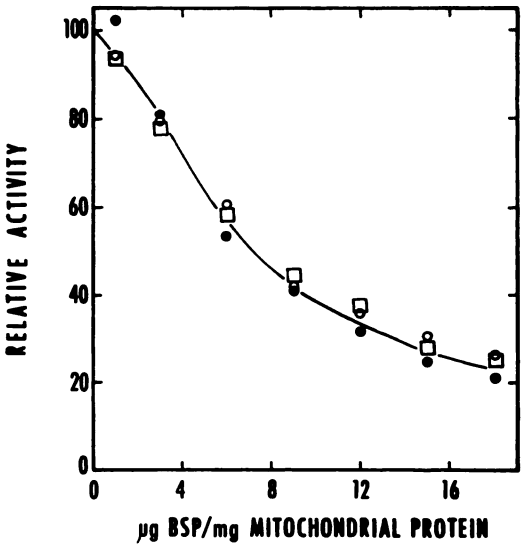

FIG. 2. *Inhibition of hepatic mitochondrial oxidative phosphorylation by BSP*

Experiments were similar to those described in Fig. 1, except that following addition of BSP 10^6 cpm of carrier-free $^{32}P_i$ were added, followed by 50 μl of 100 mM ADP. Phosphorylation was measured as indicated in the text. Substrate concentrations and control specific activities were as follows: ○, 20 mM DL-β-hydroxybutyrate, 92.7; □, 10 mM glutamate, 134; ●, 10 mM succinate, 137.

The inhibition of mitochondrial respiration by BSP is not limited to hepatic mitochondria. State 3 oxygen consumption by rat heart mitochondria in the presence of 10 mM pyruvate plus 2.5 mM L-malate (initial specific activity, 288) was inhibited 32% at 6 μg of BSP per milligram of mitochondrial protein and 56% at 24 μg/mg.

BSP also inhibited phosphorylation of ADP (Fig. 2). The degree of inhibition at each concentration of BSP was more uniform than when oxygen consumption was measured (Fig. 1). This raised the question whether BSP uncouples oxidative phosphorylation. Coupling of oxidative phosphorylation was therefore measured directly from the oxygraph tracings (Table 1).

TABLE 1

Effect of BSP on mitochondrial respiratory control and ADP to oxygen ratio

Experiments were performed as described in Fig. 1, except that state 3 respiration was initiated by 130 nmoles of ADP after depletion of endogenous substrates and addition of the indicated substrate.

Substrate	BSP	Oxygen consumption		Respiratory control ratio, state 3:state 4	ADP to oxygen ratio
		State 3	State 4		
	μg/mg mitochondrial protein	*natoms oxygen/min/mg*			*nmoles/natom*
10 mM glutamate	0	67.3	3.1	20	2.63
	1	63.2	4.4	14	2.56
	6	38.7	1.1	20	2.70
	12	17.8	3.0	6	3.08
10 mM succinate	0	95.9	6.3	15	2.00
	1	92.6	3.0	20	1.89
	6	49.9	13.6	4	2.07
	12	25.4	8.6	3	1.86
10 mM pyruvate	0	42.4	5.2	8	2.83
(+2.5 mM	1	36.8	5.0	7	2.78
L-malate)	6	22.9	5.2	4	2.65
	12	17.8	10.3	2	2.36

The results indicate that BSP does not uncouple oxidative phosphorylation. The major change with increasing BSP concentrations is a decrease in the state 3 specific activity with no consistent change in state 4 oxygen consumption or in the ADP to oxygen ratio. The respiratory control ratio also falls with increasing BSP concentrations, reflecting the decrease in state 3 rate.

Conjugated BSP. Attention was next turned to the major physiological metabolite of BSP, the BSP-GSH conjugate. The experiments shown in Table 2 indicate that 47 nmoles/mg of conjugated BSP (50 μg/mg) do not inhibit mitochondrial oxygen consumption. This is 6.5 times the molar concentration of unconjugated BSP (6 μg/mg) which caused 36% inhibition in the same experiment. Furthermore, the prior addition of conjugated BSP, GSH (50 μg/mg = 169 nmoles/mg), or cysteine (50 μg/mg = 410 nmoles/mg) to the medium failed to prevent inhibition by 6 μg/mg (7 nmoles/mg) of unconjugated BSP. This suggests that BSP is not displaced from its site of action by these compounds.

Protein binding. Earlier in this work it was noted that the extent of inhibition by a given concentration of BSP varied with the amount of mitochondrial protein added to

TABLE 2

Effect of conjugated BSP[a] and related compounds on state 3 respiration of glutamate

Experiments were performed as described in Fig. 1. The indicated compounds and BSP were added before initiation of state 3 respiration with ADP. The substrate was 10 mM glutamate.

Addition	BSP	Oxygen consumption	Percentage of control
	μg/mg mitochondrial protein	*natoms oxygen/min/mg*	
Control	0	89.4	100[b]
	6	56.8	63.5
Conjugated BSP, 25 μg/mg	0	85.2	95.3
	6	50.0	55.9
Conjugated BSP, 50 μg/mg	0	98.7	110
	6	50.5	56.4
Glutathione, 50 μg/mg	0	88.3	98.7
	6	58.3	64.5
Cysteine, 50 μg/mg	0	87.1	97.4
	6	54.1	61.5

[a] BSP-GSH thioether.

[b] This experiment was used as the basis for comparison with the other experiments.

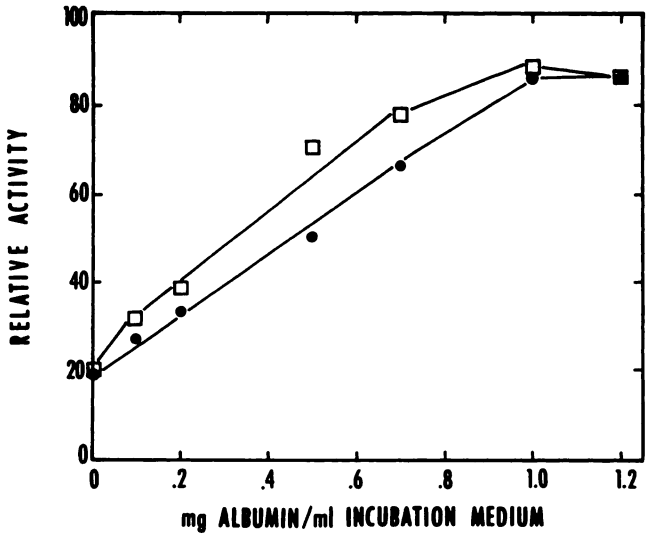

FIG. 3. *Effect of albumin concentration on BSP inhibition of oxygen consumption*
Experiments were performed as described in Fig. 1. The indicated concentrations of defatted bovine serum albumin were added to the incubation medium before the addition of 2 mg of mitochondria protein. Each point represents the rate of state 3 oxygen consumption in the presence of 10 μg of BSP per milligram of mitochondrial protein compared to a control experiment at the same concentration of albumin but lacking BSP. Substrates and specific activities in the absence of both added albumin and BSP were: ●, 10 mM succinate, 175; □, 10 mM glutamate, 96.6.

the medium, suggesting that binding of BSP to the mitochondria might be important. Since BSP is also known to be tightly bound to both serum albumin and intracellular proteins *in vivo* (23, 24), experiments were conducted to see whether the addition of protein to the incubation medium could prevent or reverse inhibition by BSP.

The inhibition by a constant amount of BSP progressively decreased with increasing concentrations of defatted bovine serum albumin in the medium (Fig. 3). The effect of albumin was not due to its previously described ability to stimulate mitochondrial respiration independently, since the control experiments for each point contained the indicated amount of albumin in the incubation medium. In addition to preventing inhibition, the addition of defatted albumin after BSP inhibition of state 3 respiration returned the rate of oxygen consumption toward the pre-BSP control level (Fig. 4). In fact, following addition of 1.0 mg of defatted albumin, the resulting rate was actually 20% greater than the control rate. The demonstration that inhibition is reversible by albumin suggests that BSP does not permanently alter mitochondrial structure.

Effect on electron transport. The ability of BSP to inhibit oxidative phosphorylation with a variety of substrates suggested a site of action of BSP which is common to the oxidation of all of the substrates. Therefore studies were performed to test the effect of BSP on some isolated steps in electron transport during oxidative phosphorylation.

BSP had no effect on the β-hydroxybutyrate (NADH), glutamate (NADH), or succinate (FAD) dehydrogenases. The succinate–cytochrome *c* reductase activity, a partial reconstitution of the site II entry into the electron transport chain, was likewise unaffected by BSP (Table 3). NADH oxidase, which measures the activity of the whole chain, was also not inhibited (Fig. 5).

In contrast, when succinate oxidase was measured in intact mitochondria, BSP clearly inhibited oxygen consumption (Fig. 5). However, when sonicated submitochondrial particles were used, exposing the inside surface of the mitochondrial inner membrane (25), there was no significant inhibition by BSP. These findings suggest that BSP may inhibit oxidative phosphorylation by an

action at the site of the mitochondrial inner membrane.

Effect of substrate concentration. One possible action of BSP at the mitochondrial inner membrane could be inhibition of substrate uptake. Therefore the effect of BSP on state 3 respiration was measured at different concentrations of ADP, P_i, and anion substrate.

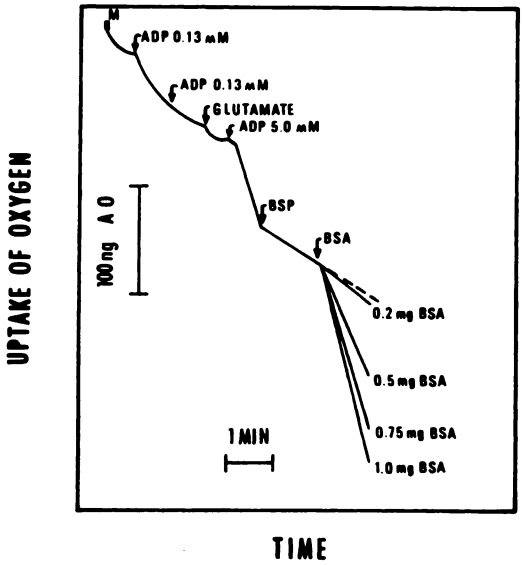

FIG. 4. *Reversal of BSP inhibition by albumin*
The figure is a composite of the oxygraph tracings of four separate experiments. At *M*, 2 mg of mitochondrial protein were added. Endogenous substrates were depleted by two successive 10-μl additions of 13 mM ADP. Then 10 μl of 1 mM glutamate were added, followed by 50 μl of 100 mM ADP. After 45 sec 20 μg of BSP were added (10 μg/mg). Defatted albumin (BSA) was added in the indicated concentrations 1.2 min later.

Varying the concentration of ADP over a 50-fold range had no effect on BSP inhibition (Table 4). This suggests that BSP is not a competitive inhibitor of adenine nucleotide translocase.

Inhibition of both heart and hepatic mitochondria by BSP did show marked sensitivity to P_i concentration (Table 5). With pyruvate plus L-malate or DL-β-hydroxybutyrate as substrate, inhibition was almost completely prevented. However, only a partial response was seen with succinate plus rotenone and palmitoyl-L-carnitine plus malate. The reason for these differing responses to P_i concentration is not clear.

The relationship between inhibition by BSP and concentration of P_i was further explored at three concentrations of BSP (Fig. 6). These data suggest that BSP may act by inhibiting uptake of P_i at the mitochondrial inner membrane.

In other experiments (not shown) state 3 respiration with glutamate was inhibited to 28% of the control rate by 20 μg of BSP per milligram at 5 mM P_i. When P_i was added to the inhibited mitochondria, raising the concentration to 10 mM, the rate of state 3 respiration rose to 49% of control. This

TABLE 3

Effect of BSP on some isolated steps of electron transport

Experiments were performed as described in the text. Specific activities were measured as nanomoles of NAD reduced per minute per milligram for glutamate and β-hydroxybutyrate dehydrogenases, as nanomoles of cytochrome *c* reduced per minute per milligram for succinate–cytochrome *c* reductase, and as nanomoles of 2,6-dichlorophenolindophenol reduced per minute per milligram for succinate dehydrogenase.

BSP	Succinate dehydrogenase	Succinate–cytochrome *c* reductase	Glutamate dehydrogenase	β-Hydroxybutyrate dehydrogenase
μg/mg mitochondrial protein	*specific activity*			
0	134	108	170	306
1	132	113	180	ND[a]
3	128	104	174	ND
6	137	110	152	302
12	143	113	180	322
18	141	100	170	322
24	130	96	170	334

[a] Not done.

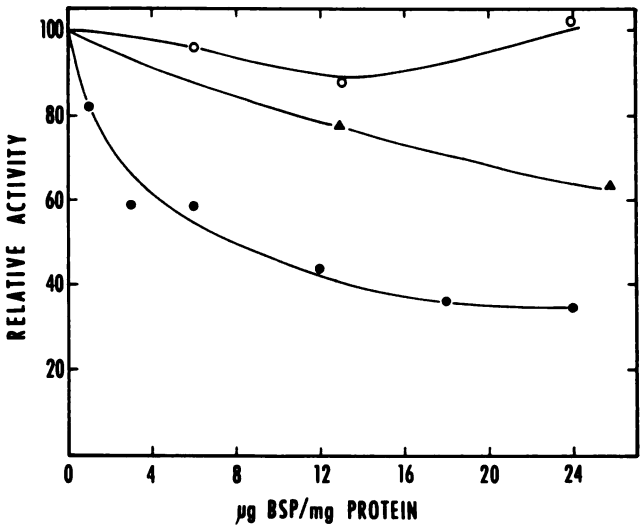

FIG. 5. *Effect of BSP on electron transport*

Methods are described in the text. O, NADH oxidase (control specific activity, 322); ●, succinate oxidase from whole mitochondria (control specific activity, 126); ▲, succinate oxidase from sonicated mitochondrial particles (control specific activity, 62.8).

TABLE 4

Effect of ADP concentration on BSP inhibition of state 3 respiration of glutamate

Experiments were conducted as described in Fig. 1, except that state 3 respiration was initiated by the indicated concentrations of ADP. The substrate was 10 mM glutamate. Control experiments contained no BSP. BSP experiments contained 15 μg of BSP per milligram of mitochondrial protein.

ADP concentration	Oxygen consumption		Percentage of control[a]
	Control	+BSP	
mM	*natoms oxygen/min/mg*		
0.1	70.0	23.6	33.8
0.2	74.4	29.3	37.9
0.5	72.6	27.1	37.3
5.0	92.2	33.6	36.4

[a] + BSP/control × 100.

change represents a reversal of inhibition rather than an effect of P_i concentration on state 3 respiration per se, since in parallel experiments an increase in P_i concentration from 5 to 10 mM resulted in less than a 5% increase in the state 3 rate of oxidation of glutamate.

When the effect of varying anion substrate concentrations on BSP inhibition was studied in the presence of 5 mM P_i (Table 6), inhibition remained constant except with succinate. Since the mitochondrial uptake of succinate has been shown to be dependent on P_i uptake (26), these data are not inconsistent with a primary relationship between BSP inhibition and P_i uptake. None of the other substrates tested, including palmitoylcarnitine plus L-malate and pyruvate plus L-malate, showed decreased inhibition with increased substrate concentration.

DISCUSSION

These experiments indicate that BSP is a potent inhibitor of oxidative phosphorylation *in vitro* in rat liver mitochondria. Results are consistent with a reversible effect of BSP on the mitochondrial inner membrane, which is prevented by increasing the concentration of either P_i or defatted albumin in the medium. P_i-sensitive inhibition of rat heart mitochondrial respiration was also shown, suggesting that the BSP effect may be applicable to other mitochondria as well.

Previous workers have shown that P_i traverses the inner membrane in exchange for hydroxyl equivalents, for dicarboxylic acids, or for mitochondrial matrix P_i (27–29). The present studies do not indicate which or how many of the P_i exchange mechanisms may be inhibited by BSP, nor

TABLE 5

Effect of varying phosphate concentration on BSP inhibition of mitochondrial respiration

Experiments were performed as described in Fig. 1, except that the P_i concentration of the incubation medium was varied as indicated. Control experiments were performed in the absence of BSP. BSP experiments contained 6 μg of BSP per milligram of mitochondrial protein.

Substrate	P_i concentration	Oxygen consumption		Percentage of control[a]
		Control	+BSP	
	mM	*natoms oxygen/min/mg*		
Liver mitochondria				
Succinate, 10 mM (+0.004 mM rotenone)	1.0	143.3	32.8	22.9
	5.0	174.0	82.9	47.6
	10.0	168.6	91.8	54.4
Palmitoyl-L-carnitine, 0.02 mM (+0.5 mM L-malate)	1.0	101.0	25.7	25.4
	5.0	131.8	52.4	39.7
	10.0	119.0	69.8	58.7
DL-β-Hydroxybutyrate, 20 mM	1.0	62.3	26.5	42.5
	5.0	58.5	44.3	75.7
	10.0	59.4	53.8	90.6
Pyruvate, 10 mM (+2.5 mM L-malate)	1.0	44.0	10.1	22.9
	5.0	48.6	38.6	78.8
	10.0	50.7	48.8	96.3
Heart mitochondria				
Pyruvate, 10 mM (+2.5 mM L-malate)	1.0	298.0	0	0
	5.0	287.9	197.8	68.7
	10.0	355.3	326.1	91.7

[a] + BSP/control × 100.

do they allow differentiation between competitive and noncompetitive inhibition. Work is under way to define further the effect of BSP on these parameters.

While the present data show toxicity *in vitro* of low concentrations of BSP, these observations cannot be extended to normal clinical situations. At the usual clinical dose, binding of BSP to serum albumin and to cytoplasmic proteins would probably prevent significant hepatic intracellular levels of unbound BSP. In addition, rapid conjugation of BSP with glutathione in normal liver would result in a noninhibitory compound. Nonhepatic tissues take up only a small fraction of intravenously administered BSP and therefore would not normally be exposed to significant levels of free BSP (30).

Whether transient inhibition of mitochondrial respiration could be shown *in vivo* if very high doses of BSP were administered, or if there were impairment of conjugation with glutathione, remains to be seen. Similarly, the effect of displacement of BSP from cytoplasmic proteins by other organic anions (31), or quantitative reduction in the levels of binding proteins by drug or disease (32), is not known.

As noted earlier, death following BSP administration has been reported in several laboratory mammals as well as in man. In the study by Curry *et al.* (7) on animals with biliary fistula, death was prevented by simultaneous perfusion of BSP and glutathione, and was not seen after perfusion with BSP-glutathione conjugate. Biliary fistula has been shown to result in abnormal BSP retention in dogs (33), possibly secondary to depletion of hepatic glutathione because of a loss of the normal enterohepatic circulation with simultaneous loss of taurine. Animals with biliary fistula also exhibit reduced bile flow after infusions of BSP,

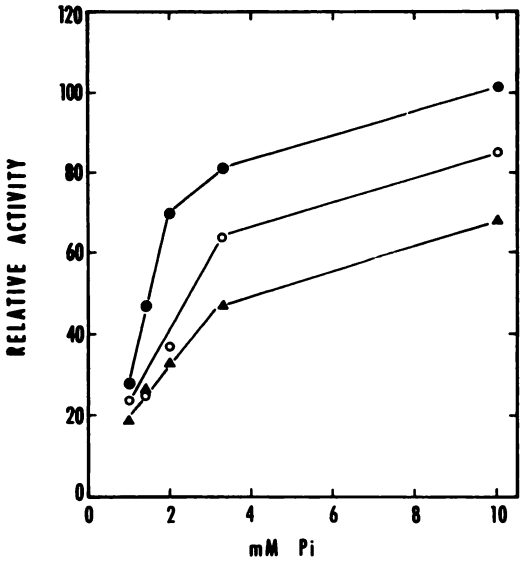

FIG. 6. *Effect of P_i concentration on BSP inhibition of oxygen consumption*

Experiments were performed as described in Fig. 1. The P_i concentration of the incubation medium was varied as shown. BSP concentrations were: ●, 3 μg/mg of mitochondrial protein; ○, 6 μg/mg; ▲, 12 μg/mg. The substrate was 10 mM glutamate; specific activity of state 3 oxygen consumption in the absence of BSP ranged between 59.3 at 10 mM P_i and 67.4 at 1 mM P_i.

but not after infusions of BSP-glutathione conjugate (7, 34). Decreased bile flow is greatest after depletion of hepatic glutathione (35). While it is possible that BSP reduces bile flow by competing with bile salt transport at the canalicular membrane, as suggested by Whelan and Combes (6), or at the sinusoidal membrane, as postulated by Curry *et al.* (7), it is also possible that BSP inhibits energy-dependent bile flow indirectly by its action on hepatic mitochondria. Further studies are necessary to explore the relationship between BSP inhibition of oxidative phosphorylation and reduction in bile flow or death in laboratory animals.

Regardless of the toxicological properties of BSP *in vivo*, the experiments presented here suggest that BSP may be an important laboratory tool in the study of oxidative phosphorylation and P_i transport across the mitochondrial inner membrane or in the study of P_i-dependent mitochondrial reactions. The concentration of BSP is readily measured in aqueous solutions. Its effect is easily reversed and probably unattended by permanent alterations in the structure or function of the mitochondrial inner membrane.

TABLE 6

Effect of varying substrate concentration on BSP inhibition of state 3 respiration

Experiments were performed as described in Fig. 1, except that the substrate concentration was varied as indicated. Control experiments contained no BSP. BSP experiments contained 6 μg of BSP per milligram of mitochondrial protein.

Substrate	Substrate concentration	Oxygen consumption		Percentage of control[a]
		Control	+BSP	
	mM	*natoms oxygen/min/mg*		
Succinate (+0.004 mM rotenone)	1.0	85.5	17.9	20.9
	5.0	103.7	40.6	39.1
	10.0	133.3	70.6	53.0
Glutamate	1.0	56.5	38.6	68.4
	5.0	67.9	52.9	77.9
	10.0	73.2	54.0	73.7
Hexanoate (+0.5 mM L-malate)	0.04	76.9	58.0	75.4
	0.20	80.4	63.0	78.4
	0.40	81.3	58.3	71.7
DL-β-Hydroxybutyrate	2.0	45.3	26.3	58.1
	10.0	54.6	33.9	62.1
	20.0	58.2	33.6	57.7

[a] + BSP/control × 100.

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