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Butylated Hydroxytoluene and Inorganic Phosphate Plus Ca^{2+} Increase Mitochondrial Permeability via Mutually Exclusive Mechanisms

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Mitochondria undergo a permeability transition (PT)[2], i.e., become nonselectively permeable to small solutes, in response to a wide range of conditions/compounds. In general, opening of the permeability transition pore (PTP) is Ca^{2+}- and P_i-dependent and is blocked by cyclosporin A (CsA), trifluoperazine (TFP), ADP, and butylated hydroxytoluene (BHT). Gudz and coworkers have reported [*7th European Bioenergetics Conference, EBEC Short Reports* (1992) **7**, 125], however, that, under some conditions, BHT increases mitochondrial permeability via a process that may not share all of these characteristics. Specifically, they determined that the BHT-induced permeability transition was independent of Ca^{2+} and was insensitive to CsA. We have used mitochondrial swelling to compare in greater detail the changes in permeability induced by BHT and by Ca^{2+} plus P_i with the following results. (1) The dependence of permeability on BHT concentration is triphasic: there is a threshold BHT concentration (ca. 60 nmol BHT/mg mitochondrial protein) below which no increase occurs; BHT enhances permeability in an intermediate concentration range; and at high BHT concentrations (>120 nmol/mg) permeability is again reduced. (2) The effects of BHT depend on the ratio of BHT to mitochondrial protein. (3) Concentrations of BHT too low to induce swelling block the PT induced by Ca^{2+} and P_i. (4) The dependence of the Ca^{2+}-triggered PT on P_i concentration is biphasic. Below a threshold of 50–100 μM, no swelling occurs. Above this threshold swelling increases rapidly. (5) P_i levels too low to support the Ca^{2+}-induced PT inhibit BHT-induced swelling. (6) Swelling induced by BHT can be *stimulated* by agents and treatments that block the PT induced by Ca^{2+} plus P_i. These data suggest that BHT and Ca^{2+} plus P_i increase mitochondrial permeability via two mutually exclusive mechanisms.

KEY WORDS: Butylated hydroxytoluene; mitochondria; permeability transition; phosphate.

INTRODUCTION

It was recognized as early as 1976 (Hunter *et al.*, 1976; Hunter and Haworth, 1979a,b; Haworth and Hunter, 1979) that when mitochondria contain Ca^{2+}, they can be induced by numerous agents to undergo a permeability transition; the inner membrane (i.m.) becomes permeable to solutes smaller than 1500 Da. Although the triggering agents are chemically disparate, the transitions that they induce have several key properties in common. In all cases, with the possible exception of phenylarsine oxide (Lenartowicz *et al.*, 1991), matrix Ca^{2+} is absolutely required. In addition,

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[2] Abbreviations: BHT, butylated hydroxytoluene; CsA, cyclosporin A; F-CCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; DTT, dithiothreitol; i.m., inner mitochondrial membrane; NEM, *N*-ethylmaleimide; PCoA, palmitoyl coenzyme A; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, polyethylene glycol; P_i, inorganic phosphate; PT, permeability transition; PTP, permeability transition pore; TBH, *t*-butyl hydroperoxide; TFP, trifluoperazine.

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the process is sensitive to CsA, TFP, ADP, and, generally, to BHT (for a review see Gunter and Pfeiffer, 1990).

Butylated hydroxytoluene has been relatively widely studied as an inhibitor of the mitochondrial permeability transition. It has been demonstrated to block transitions induced by phenylarsine oxide, arsenate, diamide, palmitoyl coenzyme A (PCoA), Ca^{2+}, Fe^{2+} plus ascorbate, Adriamycin aglycones, cumene hydroperoxide, and *t*-butylhydroperoxide (TBH) (Novgorodov *et al.*, 1987; 1989; Lenartowicz *et al.*, 1991; Carbonera and Azzone, 1988; Wolkowicz, 1988; Sokolove, 1990; Gogvadze and Zhukova, 1991; Gogvadze *et al.*, 1992).

In general, interest in BHT has focused on its antioxidant function and the possibility that it might be used to evaluate the role of lipid peroxidation in transition induction. The conclusion has been that neither lipid (Novgorodov *et al.*, 1989; 1991) nor glutathione (Carbonera and Azzone, 1988) oxidation is tightly linked to PT occurrence. Several features suggested, however, that inhibition of the PT by BHT might be of wider interest. The compound blocked transition induction by Adriamycin aglycones in liver but not in heart mitochondria (Sokolove, 1990). Furthermore, although BHT blocked the PT induced by PCoA when P_i was present, BHT was reported to *induce* the transition when P_i was omitted from the assay medium. The ability of P_i to abrogate BHT-triggered swelling was eliminated by ruthenium red and mersalyl (Wolkowicz, 1988). Finally, BHT discriminated to some extent among triggers of the PT. Its inhibitory effects on the TBH-triggered transition were partially overcome when the TBH concentration was increased, while increases in diamide or Ca^{2+} concentration were without effect on BHT inhibition of the diamide- and Ca^{2+}-induced transitions (Carbonera and Azzone, 1988). A second report of the ability of BHT to trigger the PT in a P_i-sensitive fashion (Gudz *et al.*, 1992) sparked our interest in the compound.

Inorganic phosphate appears to play a special role in the mitochondrial PT. Only one triggering agent, again, phenylarsine oxide, is known to induce the transition in the absence of P_i (Novgorodov *et al.*, 1987). In contrast, diamide (Novgorodov *et al.*, 1987; Carbonera and Azzone, 1988), TBH (Carbonera and Azzone, 1988; Lapidus and Sokolove, 1994), arsenate (Novgorodov *et al.*, 1987), PCoA, acetoacetate (Wolkowicz and McMillan-Wood, 1980), carboxyatractylate (Le Quoc and Le Quoc, 1988), and Ca^{2+} (Lapidus and Sokolove, 1994) all appear to induce the PT only when P_i is present. The interaction between BHT and P_i, suggested by the data of Wolkowicz cited above and by the preliminary report of Gudz and coworkers (1992), indicated that a thorough examination of the effects of BHT and P_i on the mitochondrial PT might prove fruitful.

The experiments reported here demonstrate a unique and complex relationship between BHT and P_i. Each agent can increase mitochondrial permeability, although, in the case of P_i, Ca^{2+} must also be present. For each agent there is a threshold concentration below which permeability does not increase; above this threshold, induction appears to be a highly cooperative phenomenon. At concentrations below those required to trigger swelling, each agent inhibits the swelling induced by the other, acting specifically to increase the threshold for transition induction. Finally, some compounds that inhibit the transition induced by P_i plus Ca^{2+} can stimulate the BHT-induced process. We interpret these findings to indicate that the two triggering conditions increase mitochondrial permeability via mutually exclusive mechanisms and to suggest the presence of a molecular "switch," sensitive to BHT and P_i, that determines which of these mechanisms will be operative. In the discussion that follows, both BHT and Ca^{2+} plus P_i are referred to as inducing a PT. It is clear that both conditions increase mitochondrial permeability; it is not clear, however, whether both operate on a single pore, i.e., the PTP. Some of these results were presented earlier in preliminary form (Sokolove and Haley, 1995).

MATERIALS AND METHODS

Mitochondria were isolated from the livers of large (>250 g), male Sprague-Dawley rats by standard differential centrifugation techniques in low-salt buffers (Lapidus and Sokolove, 1993). Assays were carried out at 30°C in a resin (Chelex-100)-treated reagent containing 210 mM mannitol, 70 mM sucrose, and 5 mM Hepes-KOH, pH 7.4, supplemented with 0.8 μM rotenone. Unless noted otherwise, the mitochondrial protein concentration was 0.4 mg/ml, determined according to Lowry *et al.* (1951) using BSA as standard.

Increases in mitochondrial permeability were followed via mitochondrial swelling by monitoring apparent absorbance at 540 nm (A_{540}; Tedeschi and Harris, 1955) with an LKB Ultrospec II UV-Visible spectrophotometer. Mitochondria were incubated for 3 min

in the basic assay reagent. When Ca^{2+} and P_i were used to trigger the transition, P_i was added prior to the mitochondria, Ca^{2+} was added at 3 min, and the mitochondria were energized with 5 mM succinate at 3.5 min. When BHT was the trigger, succinate was added at 3.5 min and BHT at 5 min. *N*-Ethylmaleimide (NEM)-treatment was carried out by incubating mitochondria for 2 min on ice in the presence of 50 nmol NEM/mg protein, then adding them to the assay reagent. Other inhibitors and test compounds were added to the assay reagent prior to the mitochondria. Data were quantified in terms of the maximal rate of decrease of A_{540} (ΔA_{540}/min/mg) by drawing a tangent to the plot of absorbance vs. time at its steepest point. As observed by Petronilli and coworkers (1993), the rate of decrease in A_{540} is proportional to the rate at which mitochondria are recruited to the transition. Respiration was measured under the identical conditions with a Clark-type electrode (Estabrook, 1967). Results are representative of multiple ($\geq$3) experiments unless specified otherwise.

Cyclosporin A (OL 27-400) was the generous gift of Sandoz Research Institute (East Hanover, New Jersey). BHT was purchased from Calbiochem (La Jolla, California) and was made up fresh as a 5 mM solution in 95% ethanol early on the day of each experiment to permit it to dissolve completely. Chelex-100 was from Bio-Rad (Richmond, California); the remaining biochemicals were from Sigma Chemical Company (St. Louis, Missouri). All other reagents were of the highest quality available.

RESULTS

In their preliminary report, Gudz and coworkers (1992) suggested that BHT was an inhibitor of the mitochondrial PT at concentrations between 1 and 20 μM and an inducer of the transition at concentrations between 40 and 100 μM. That observation is confirmed and extended in Figs. 1–3. Mitochondrial permeability was monitored via both mitochondrial swelling (Fig. 1A) and respiration (Fig. 1B), and its dependence on BHT concentration was found to be triphasic. By both measures, BHT was without obvious effect when its concentration was below 50–60 nmol/mg mitochondrial protein. As the BHT concentration was increased above this threshold level, swelling was stimulated dramatically and state 4 respiration was increased, both of which indicate increased i.m. permeability. The sharpness of the increase in swelling rate with increasing BHT concentration suggested that the interaction was highly cooperative. At BHT levels in excess of ca. 125 nmol/mg, swelling decreased as did both state 4 respiration and respiration uncoupled by F-CCP. These findings are in complete agreement with earlier reports (Thompson and Moldeus, 1988; Fereira, 1990) that BHT increased respiration, elicited Ca^{2+} release, and decreased membrane potential, i.e., induced a PT, at concentrations below 100 nmol/mg, while BHT and its analog butylated hydroxyanisole inhibited electron transport at higher concentrations. Apparent differences in the concentration dependence of BHT effects in various publications can be eliminated by normalizing

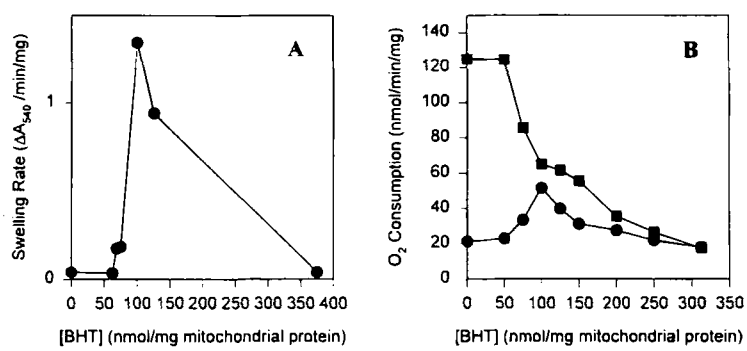

Fig. 1. Dependence of induction of the mitochondrial permeability transition on BHT concentration. (A) Effect of BHT concentration on the maximal rate of mitochondrial swelling. (B) Effect of BHT concentration on succinate-supported respiration measured in the presence (■) and in the absence (●) of 400 nM F-CCP. Results shown in (A) and (B) were obtained with two different preparations of mitochondria. Data of the sort shown in (A) were obtained routinely; the experiment shown in (B) was one of two yielding virtually identical results. Experimental details were as outlined in Materials and Methods.

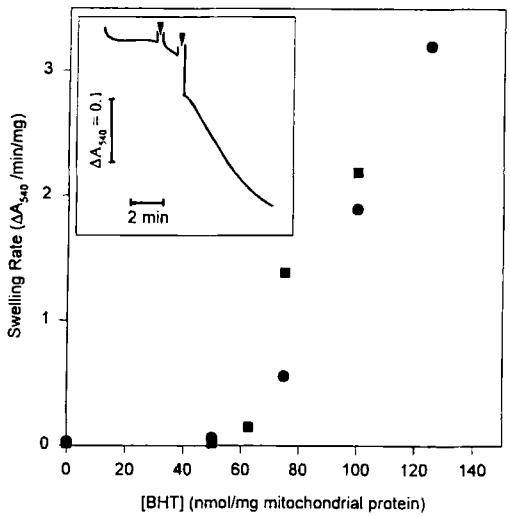

Fig. 2. Dependence of BHT-induced swelling on the ratio of BHT to mitochondrial protein. BHT-induced swelling was measured at two protein concentrations, 0.2 (●) and 0.4 (■) mg/ml. One experiment representative of three. Inset: time course of swelling induced by 35 μM BHT. Succinate was added at the first arrow, BHT at the second.

BHT levels to mitochondrial protein concentration. As shown in Fig. 2, it is the ratio of BHT concentration to mitochondrial protein that determines BHT effectiveness as an inducer of the PT.

A trace depicting the effect of 35 μM BHT on A_{540} is shown as an inset to Fig. 2. An instantaneous decrease in absorbance is followed by a slower decrease indicative of mitochondrial swelling. The instantaneous decrease in A_{540}, which was proportional to BHT concentration, i.e., demonstrated no threshold (data not shown), may reflect partitioning of BHT into the mitochondrial membranes. Clement and Gould

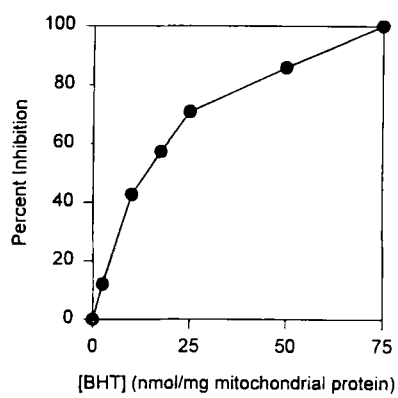

Fig. 3. Dependence of inhibition of the swelling induced by Ca^{2+} plus P_i on BHT-to-protein ratio. Swelling was induced with 1 mM P_i and 100 μM Ca^{2+}. The control swelling rate (no BHT) was ΔA_{540}/min/mg = 2.143.

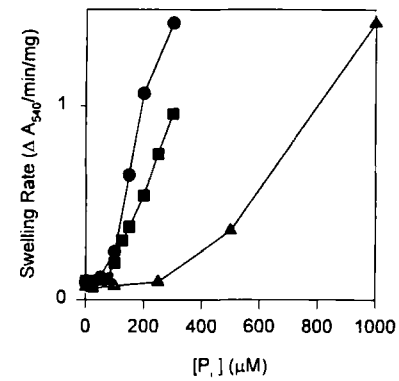

Fig. 4. Dependence of the maximal rate of Ca^{2+}-induced swelling on P_i concentration. Data shown were obtained in a single experiment at three different Ca^{2+} concentrations: 60 μM (▲), 123 μM (■), and 200 μM (●). They are representative of multiple (>3) experiments.

(1980) demonstrated, using a variety of membranes, that the mole fraction of BHT in a membrane is directly proportional to the concentration of BHT in the suspending medium.

The effect of BHT on the PT induced by Ca^{2+} and P_i is shown in Fig. 3. In this experiment, swelling was induced with 1 mM P_i and 100 μM Ca^{2+}. Inhibition by BHT was essentially complete within the threshold for BHT-induced swelling.

The ability of P_i to support Ca^{2+}-induced swelling is examined in Fig. 4. Regardless of the Ca^{2+} concentration, there is a P_i concentration below which the PT fails to occur. In our experiments, that threshold concentration was on the order of 50–100 μM. P_i concentrations that fell below this level were, however, sufficient to eliminate BHT-induced swelling (Fig. 5),

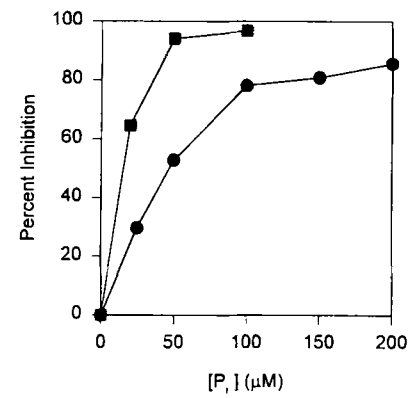

Fig. 5. Dependence of inhibition of BHT-induced swelling on P_i concentration. Data are from two different experiments using 40 μM BHT (■; control swelling rate was ΔA_{540}/min/mg = 1.343) and 50 μM BHT (●; control swelling rate was ΔA_{540}/min/mg = 1.429), respectively.

just as BHT levels too low to induce swelling were able to block P_i-induced swelling (Fig. 3). P_i also eliminated the effect of low BHT concentrations on respiration, i.e., increased the threshold BHT concentration required to enhance State 4 and inhibit uncoupled respiration (data not shown). A more extensive investigation focussing on swelling (Fig. 6) demonstrated not only that P_i increased the threshold BHT concentration required for PT induction (Fig. 6A) but also that BHT increased the minimal P_i concentration needed for swelling (Fig. 6B). This relationship was unique. Other inhibitors of the mitochondrial PT [ADP + oligomycin, CsA, TFP, dithiothreitol (DTT)] were not themselves able to trigger the transition, and other triggers (Ca^{2+}, diamide, *t*-butylhydroperoxide) failed to inhibit BHT-induced swelling (data not shown).

On the basis of sizing experiments using polyethylene glycol (PEG), Gudz *et al.* (1992) suggested that the pore being opened by BHT was the traditional PTP. It could therefore be argued that BHT and Ca^{2+} plus P_i are inducing a single pore to open but are doing so in different ways. To test this hypothesis, the effects of agents known to inhibit the traditional PTP on the PTs induced by BHT- and Ca^{2+} plus P_i were compared (Table I).

Not all inhibitors blocked the *traditional* PT equally effectively. When the PT was induced by Ca^{2+} (100 μM) plus P_i (1 mM), EGTA, CsA, Mg^{2+}, and NEM (50 nmol/mg) were potent inhibitors, while DTT and the combination of ADP and oligomycin inhibited poorly. The apparently poor inhibition exerted by TFP reflects the transience of inhibition by this agent (Broekemeier and Pfeiffer, 1989). Although swelling was greatly delayed, it proceeded ultimately at a substantial rate. This finding is not unexpected. We have reported (Lapidus and Sokolove, 1994) that ADP plus oligomycin is strongly inhibitory only when the PT is triggered by P_i. In the experiments shown here, P_i facilitated the transition, but the primary trigger was Ca^{2+}. Similarly, Costantini and coworkers (1995) have recently reported that monobromobimane, a bifunctional reagent capable of forming stable thiol adducts, can interfere with the PT when the triggering agent targets sulfhydryls but not when the trigger is Ca^{2+}.

Table I also suggests that inhibitors of the traditional PT can *stimulate* BHT-induced swelling. TFP provides the most dramatic example of such stimulation. Although TFP itself had no effect on mitochondrial volume or respiration (data not shown), in the presence of TFP, BHT induced swelling that was generally too rapid to measure. TFP stimulated at both high (60 μM) and low (35 μM) concentrations of BHT. In contrast, ADP plus oligomycin stimulated only when BHT was limiting (Table I and Fig. 7). In our experience, after TFP, NEM treatment was the next most robust stimulant of BHT-induced swelling, followed by the combination of ADP plus oligomycin. CsA and DTT were weaker enhancers, and in fact, stimulation was not always observed with these agents.

The results in Table I support the proposition (Gudz *et al.*, 1992) that Ca^{2+} is not required for the BHT-induced transition. Furthermore, they suggest that, at limiting BHT concentrations, energization swelling, whereas it inhibits stimulatory when BHT is present in excess.

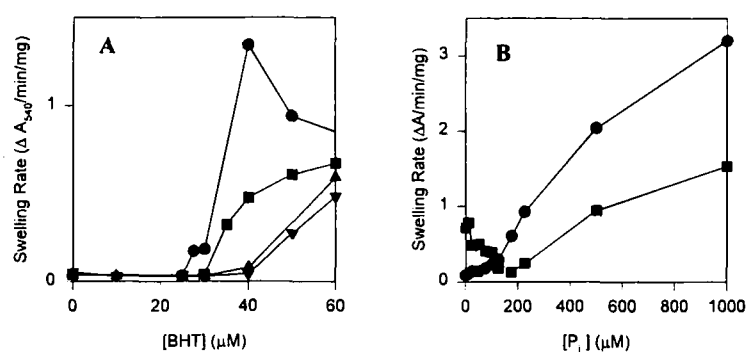

Fig. 6. Reciprocal effects of BHT and P_i on the mitochondrial PT. (A) Effect of P_i on the ability of BHT to induce mitochondrial swelling. The dependence of swelling rate on BHT concentration was measured in the presence of 20 (■), 50 (▲), and 100 (▼) μM P_i and in its absence (●) (B) Effect of BHT on the ability of P_i to induce mitochondrial swelling in the presence of Ca^{2+}. The Ca^{2+} concentration was 200 μM. BHT concentration was either zero (●) or 20 μM (■).

Table I. Effect of Inhibitors of the Mitochondrial Permeability Transition on Swelling Induced by BHT and by P_i + Ca^{2+}

Addition	1 mM P_i + 100 μM Ca^{2+}		35 μM BHT		60 μM BHT	
	ΔA/min/mg[a]	V^+/V^-[b]	ΔA/min/mg	V^+/V^-	ΔA/min/mg	V^+/V^-
Control	3.73	NA	0.115 ± 0.019 (4)[c]	NA	2.02 ± 0.25 (3)[c]	NA
143 μM ADP + 1.88 μg/ml oligomycin	1.37	0.367	0.443	3.85	1.64	0.81
60 nM CsA	0.056	0.015	0.146	1.27	2.80	1.39
50 μM TFP	0.472	0.127	3.13	27.2	TRTM[d]	>>1
0.5 mM EGTA	0	0	0.109	0.95	1.98 (2)[c]	0.98
5 mM $MgCl_2$	0.007	0.002	0.328	2.85		
NEM treatment (50 nmol/mg)	0.066	0.018	0.527	4.58	2.10	1.04
2 mM DTT	2.55	0.684	0.209	1.82	2.71	1.34
Omit succinate	.397	0.106	0.259	2.25	0.803	0.398

[a] Swelling rates were calculated as the maximal rate observed, corrected for the swelling rate measured in the absence of triggering agent.
[b] Swelling rate in the presence of agent/Swelling rate in the absence of agent, except in the case of succinate ommission where the ratio is inverted.
[c] Average of the number of measurements indicated in parentheses.
[d] Too rapid to measure.

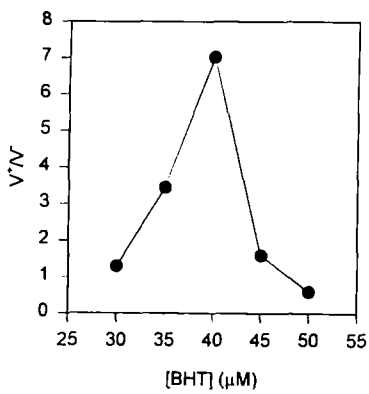

Fig. 7. Stimulation of BHT-induced swelling by ADP and oligomycin. ADP (143 μgM) and oligomycin (1.88 μ/ml), when present, were added prior to the mitochondria. The ratio of the maximal swelling rate obtained in the presence of ADP plus oligomycin to the maximal swelling rate obtained in their absence (V^+/V^-) is plotted as a function of BHT concentration. Although the extent of stimulation by ADP plus oligomycin was variable (1.75 to 7.02-fold in three experiments), the pattern was invariant.

DISCUSSION

The observations presented above document the reciprocal effects of BHT and inorganic phosphate on mitochondrial permeability. These two agents may open or contribute to opening a single PTP via two alternative mechanisms or may increase mitochondrial permeability via two distinct, but mutually exclusive, routes. Several observations argue for involvement of a single pore. The pores opened by the two agents are both influenced by a group of relatively selective compounds (Table I) including TFP, NEM, ADP, and CsA, but the effects of these compounds are complementary: the P_i-dependent pore is inhibited, while the BHT-induced pore can be stimulated. Furthermore, the effects of BHT and P_i appear to be antagonistic (Fig. 6); each increases the concentration of the other required to induce swelling. Finally, Gudz *et al.* (1992) have suggested that the pores opened by the two agents are the same because both exclude moderate sized PEG. However, in our hands, the sizing protocol of Pfeiffer and coworkers (1995) indicates that the BHT-induced pore is substantially larger than the pore opened by Ca^{2+} plus P_i (data not shown), a finding suggesting that the BHT-induced pore and the PTP may not represent a single chemical entity.

The complex dependence of PT occurrence on P_i concentration suggests that P_i acts via more than one mechanism. Rottenberg and Marbach (1990) reported stimulation of Ca^{2+} uptake and enhancement of Ca^{2+} buffering by brain mitochondria at P_i concentrations below 200 μM. These authors attributed the P_i effect to P_i uptake via the phosphate translocator and precipitation of calcium phosphate in the mitochondrial matrix with resultant stimulation of Ca^{2+} accumulation. Zoccarato and Nicholls (1982) have provided convincing evidence that phosphate does decrease the matrix free Ca^{2+} concentration. We could, by extension, conclude that P_i exerts an inhibitory effect on the PT by decreasing the free Ca^{2+} concentration in the

mitochondrial matrix and that this effect would be observed at low (<200 μM) P_i concentrations. We have reported (Lapidus and Sokolove, 1994) that P_i can induce the transition by facilitating the efflux of matrix ADP, which is a physiological inhibitor of the transition. The P_i concentrations required to drive adenine nucleotide efflux from liver mitochondria (Austin and Aprille, 1984) or to trigger the PT (Lapidus and Sokolove, 1994) fall in the millimolar range. It can therefore be proposed that the threshold concentration of P_i required to support the Ca^{2+}-dependent PT represents the concentration at which the latter effect, i.e., P_i-induced ADP efflux and the consequent PT induction, becomes dominant. This explanation is insufficient, however, to account for the ability of P_i to inhibit the permeability increase triggered by BHT, as BHT-induced swelling appears to be unaltered by the presence of 0.5 mM EGTA (Table I).

The mechanism of BHT action must remain a matter of speculation. Again, the complex dependence of mitochondrial responses on BHT concentration suggests that the compound may be exerting more than one effect. Several investigators (Novgorodov *et al.*, 1989; 1991; Carbonera and Azzone, 1988) have argued that the inhibitory effects of BHT on the mitochondrial PT are unrelated to its antioxidant activity. We propose that the possible effects of this agent on the lipid environment of the pore be given serious consideration. Cheng and coworkers (1987) reported that BHT at 23 mol. % induced the hexagonal H_{II} phase in lipid mixtures consisting of 50% soy phosphatidylethanolamine (PE) and 50% egg phosphatidylcholine (PC). BHT has been found to lower the temperature of onset of the gel to lamellar phase transition and to broaden the transition substantially (Cheng and Lepock, 1985), an effect consistent with BHT positioning itself in the membrane just below the polar head group region and near the top of the fatty acyl chains (Jain and Wu, 1977). Such behavior would be expected to favor the hexagonal H_{II} phase.

If we use the value of Fleischer *et al.* (1967) of 0.18 mg phospholipid/mg liver mitochondrial protein and assume a molecular weight of 750 for the typical phospholipid, we can calculate that the phospholipid concentration in our assays was 96 μM, while the BHT concentration required to induce the PT was on the order of 20 μM (50 nmol/mg × 0.4 mg/ml), i.e., the threshold BHT concentration for induction of the PT was 21 mol. %. The mole fractions of PE and PC in the mitochondrial inner membrane are approximately equal (45%); 10 mol. % is contributed by cardiolipin (Fleischer *et al.*, 1967; Daum, 1985). Thus, the inner membrane closely resembles the artificial membranes examined by Cheng *et al.* (1987), and BHT induces a permeability transition in the inner membrane at approximately the same mol. % observed to induce the hexagonal H_{II} phase in the model system. Involvement of inverted lipid phases in regulation of mitochondrial permeability has been proposed by others (Wolkowicz, 1988; Sciamanna *et al.*, 1992). Viewed most simply, major alterations in lipid organization could relax structural constraints maintaining the pore in its closed conformation. This model might also be consistent with a change in pore dimensions in the presence of BHT. If this were the case, BHT and Ca^{2+} plus P_i might after all act on a single pore, namely, the traditional PTP.

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