

Oxidative Stress Is Responsible for Mitochondrial Permeability Transition Induction by Salicylate in Liver Mitochondria*

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The interaction of salicylate with the respiratory chain of liver mitochondria generates hydrogen peroxide and, most probably, other reactive oxygen species, which in turn oxidize thiol groups and glutathione. This oxidative stress, confirmed by the prevention of action by antioxidant agents, leads to the induction of the mitochondrial permeability transition in the presence of Ca^{2+} . This phenomenon induces further increase of oxidative damage resulting in impairment of oxidative phosphorylation and β -oxidation, cardinal features of Reye's syndrome in the liver. Mitochondrial permeability transition induction also induces the release of cytochrome *c* and apoptotic inducing factor from mitochondria, suggesting that salicylate also behaves as a pro-apoptotic agent. The reactive group of salicylate for inducing oxidative stress is the hydroxyl group which, by interacting with a Fe-S cluster of mitochondrial Complex I, the so-called N-2(Fe-S) center, produces reactive oxygen species.

Salicylates and non-steroid drugs such as sodium salicylate, aspirin, and indomethacin, are widely prescribed for treating inflammation (1). Their efficacy is, at least partially, attributed to their ability to inhibit cyclooxygenase (2, 3). In this regard, blockade of cyclooxygenase activity is believed to be linked to the chemopreventive effect of salicylates (4, 5). Data from other studies also indicate that cyclooxygenase-2 inhibitors increase the susceptibility of cancer cells to apoptosis (6, 7). Other reports propose the involvement of a cyclooxygenase-independent pathway during apoptosis signaling (8, 9). In addition, it has been shown that aspirin induces activation of NF- κ B, which is required for the anti-tumor effects of aspirin (10). It is the general opinion, supported by a number of reports, that apoptosis is mediated by the phenomenon of mitochondrial permeability transition (MPT)² (for reviews, see Refs. 11 and 12).

Salicylates have damaging effects on isolated mitochondria, causing the uncoupling of oxidative phosphorylation and swelling (13–15). Aspirin and salicylate affect mitochondrial calcium homeostasis and act synergistically with the cation to impair mitochondrial respiration and ATP synthesis (16, 17). Again, salicylates inhibit the Krebs cycle enzyme α -ketoglutarate dehydrogenase (18).

Salicylates are in fact able to induce MPT in the presence of Ca^{2+} in isolated liver mitochondria (19) but also in "in situ" mitochondria of

cultured hepatocytes (12). These observations indicate that the onset of MPT by salicylate and Ca^{2+} represents the pathological mechanism causing mitochondrial injury in Reye's syndrome, a lethal disorder occurring in children, generally after a preliminary viral infection. Epidemiological and experimental evidence demonstrate a close correlation between Reye's syndrome and the use of aspirin (20, 21). The main cause of this syndrome is most probably a primary mitochondrial injury. Brain and liver mitochondria exhibit morphologic alterations such as matrix swelling, decreased matrix density, and loss of cristae (22), typical of organelles which have undergone a permeability transition.

A broad range of oxidant agents, thiol reagents, heavy metals, and uncouplers are able to trigger MPT in isolated mitochondria in the presence of supraphysiological Ca^{2+} concentrations (for reviews, see Refs. 23 and 24). The immunosuppressive cyclic endecapeptide, cyclosporin A (CsA) specifically blocks the phenomenon (25, 26), which is because of the opening of a pore, identified as a non-selective, highly conductive "megachannel" (24). Opening of the transition pore causes a series of events culminating in bioenergetic collapse and a redox catastrophe (11).

The mechanism by which salicylate induces MPT has not yet been elucidated, although some models have been proposed. Salicylate may bind directly to the pore-forming structure, promoting pore opening (12). Alternatively, the drug may intercalate into the mitochondrial membrane, altering its surface potential and thereby decreasing the gating potential of the pore (27). Other authors have proposed that the induction of MPT in tumor cells is because of oxidative stress following the production of reactive oxygen species (ROS), elicited by salicylate through a Rac1-NADPH oxidase-dependent pathway (28).

Taking into account this capacity of salicylate to generate ROS at cytosol level, and recent observations that the interaction of the isoflavonoid genistein with the mitochondrial electron transport chain by means of its hydroxyl group in 4'-position, also generates ROS (29), this study is aimed at determining whether salicylate, by interacting with respiratory complexes, can induce the oxidative stress responsible for MPT induction in isolated liver mitochondria. Another aim was to identify the specific component(s) of the respiratory chain that constitutes the target(s) of salicylate, to propose the reaction mechanism(s) of ROS production.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal antibody anti-cytochrome *c* (Cyt *c*) was purchased from Pharmingen, rabbit polyclonal antibody anti-apoptosis-inducing factor (AIF) was purchased from Chemicon International. All other reagents were of the highest purity commercially available.

Mitochondrial Isolation and Standard Incubation Procedures—Rat liver mitochondria (RLM) were isolated by conventional differential

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² The abbreviations used are: MPT, mitochondrial permeability transition; AIF, apoptosis inducing factor; BKA, bongkrekic acid; CsA, cyclosporin A; Cyt *c*, cytochrome *c*; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; RLM, rat liver mitochondria; ROS, reactive oxygen species; $\Delta\Psi$, membrane potential.

centrifugation in a buffer containing 250 mM sucrose, 5 mM HEPES (pH 7.4), and 1 mM EGTA (30); EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as a standard (31).

Mitochondria (1 mg protein/ml) were incubated in a water-jacketed cell at 20 °C. The standard medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 50 μM Ca^{2+} , and 1.25 μM rotenone. Variations and/or other additions are given with each experiment.

Determination of Mitochondrial Functions—Membrane potential ($\Delta\Psi$) was calculated on the basis of movement of the lipid-soluble cation tetraphenylphosphonium through the inner membrane, measured using a tetraphenylphosphonium-specific electrode (32). Mitochondrial swelling was determined by measuring the apparent absorbance change of mitochondrial suspensions at 540 nm in a Kontron Uvikon model 922 spectrophotometer equipped with thermostatic control.

Oxygen uptake was measured by a Clark electrode. This measurement allowed calculation of the rate of state 3 (ADP-stimulated) respiration (V_3), the rate of state 4 (non-ADP-stimulated) respiration (V_4), the respiratory control index ($\text{RCI} = V_3/V_4$) and the phosphate to oxygen ratio (ATP generated divided by oxygen consumed in state 3). ATP synthesis was measured by the enzymatic method of Lemasters and Hackenbrock (33). Adenine nucleotide efflux was monitored according to Ross *et al.* (34). The redox state of endogenous pyridine nucleotides was followed fluorometrically in an Aminco-Bowman 4-8202 spectrofluorometer, with excitation at 354 nm and emission at 462 nm. Efflux of oxidized pyridine nucleotides was detected according to Carpenter and Kodicek (35). The production of H_2O_2 in mitochondria was measured fluorometrically by the scopoletin method (37) in an Aminco-Bowman 4-8202 spectrofluorometer.

It should be noted that the measured amount of H_2O_2 refers to that diffusing out of mitochondria, as the enzyme for its determination (horseradish peroxidase) cannot enter mitochondria. This measurement gives a qualitative indication but cannot be considered for rigorous quantitative evaluations.

β -Oxidation of palmitoylcarnitine was assayed by the following procedure. RLM (1 mg/ml) were incubated for 10 min in standard medium, in the conditions indicated in the specific figure legend. Then 1-ml portions of the suspensions were withdrawn and centrifuged in an Eppendorf bench centrifuge (model 5415C) at 15,000 rpm. The supernatants were discarded, and mitochondrial pellets were resuspended with 30 μl of isolation medium. Subsequently RLM were incubated in the water-jacketed cell at 20 °C for oxygen uptake measurement. The assay medium contained 100 mM KCl, 20 mM Hepes (pH 7.4), 5 mM potassium-phosphate, 0.1 mM EGTA, bovine serum albumin (0.5 mg/ml), 2.5 mM oxalacetate, 0.5 mM ADP, and 30 μM palmitoylcarnitine. The protein sulfhydryl oxidation assay was performed according to Santos *et al.* (36). The oxidation of glutathione was performed as in Tietze (38). The redox state of endogenous pyridine nucleotides was followed fluorometrically in an Aminco-Bowman 4-8202 spectrofluorometer with excitation at 354 nm and emission at 462 nm.

Detection of Cyt c and AIF Release—Mitochondria (1 mg of protein/ml) were incubated for 15 min at 20 °C in standard medium with the appropriate additions. The reaction mixtures were then centrifuged at $13,000 \times g$ for 10 min at 4 °C to obtain mitochondrial pellets. The supernatant fractions were further spun at $100,000 \times g$ for 15 min at 4 °C to eliminate mitochondrial membrane fragments and concentrated five times by ultrafiltration through Centrikon 10 membranes (Amicon) at 4 °C. Aliquots of 10 μl of the concentrated supernatants were subjected to 15% SDS-PAGE for Cyt c and 10% SDS-PAGE for AIF and analyzed by Western blotting using mouse anti-Cyt c antibody and rabbit anti-AIF antibody.

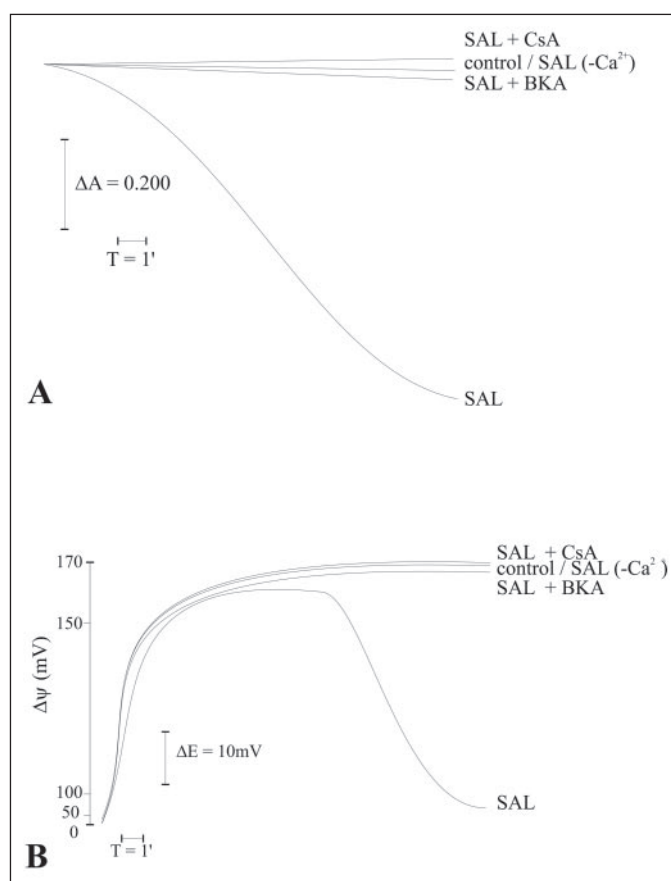


FIGURE 1. Mitochondrial swelling (A) and $\Delta\Psi$ collapse (B) induced by salicylate in rat liver mitochondria. Effect of cyclosporin A (CsA) and bongkreik acid (BKA). RLM were incubated in standard medium in conditions described under "Experimental Procedures." Salicylate (SAL) was present at 0.5 mM concentration. When added to the medium, 1 μM CsA or 5 μM BKA were present. Control traces refer to RLM incubated in standard medium or in standard medium deprived of Ca^{2+} in presence of salicylate. Downward deflection (A), mitochondrial swelling. ΔE value (B), electrode potential. Both assays were performed four times, with comparable results.

RESULTS

The results shown in Figs. 1 and 2 summarize the well known effects of salicylate in inducing MPT, as also previously reported by other authors (*e.g.* Ref. 39). Fig. 1A shows that energized RLM, incubated in standard medium in the presence of 50 μM Ca^{2+} , when treated with 0.5 mM salicylate undergo extensive swelling, evidenced by a strong decrease in the apparent absorbance of the mitochondrial suspension. This colloid-osmotic alteration is accompanied by rapid and almost complete collapse of $\Delta\Psi$ (Fig. 1B). Fig. 2 shows other events closely correlated with the above observed swelling and $\Delta\Psi$ collapse. Fig. 2A shows that salicylate enhances O_2 consumption with both succinate (*a*) and β -hydroxybutyrate (*b*) as substrates. However, in the latter case, respiration is completely blocked after 7–8 min of incubation. The same panel also shows respiratory control index and phosphate to oxygen ratio calculations, obtained by succinate oxidation after 10 min of incubation, that is, in conditions of pore opening. Salicylate is also able to provoke the efflux of adenine nucleotides (Fig. 2B) and of oxidized pyridine nucleotides (Fig. 2C). Fig. 2D shows the effect of salicylate-induced MPT on β -oxidation. This determination, like that of phosphorylation parameters, was performed during the opening of the transition pore. The results show palmitoylcarnitine oxidation is completely inhibited in the presence of salicylate.

The concentration of salicylate used, 0.5 mM, is of the same order of magnitude as that reached in the cytosol of hepatic cells after aspirin therapy (12, 40). All the observed events are completely prevented by

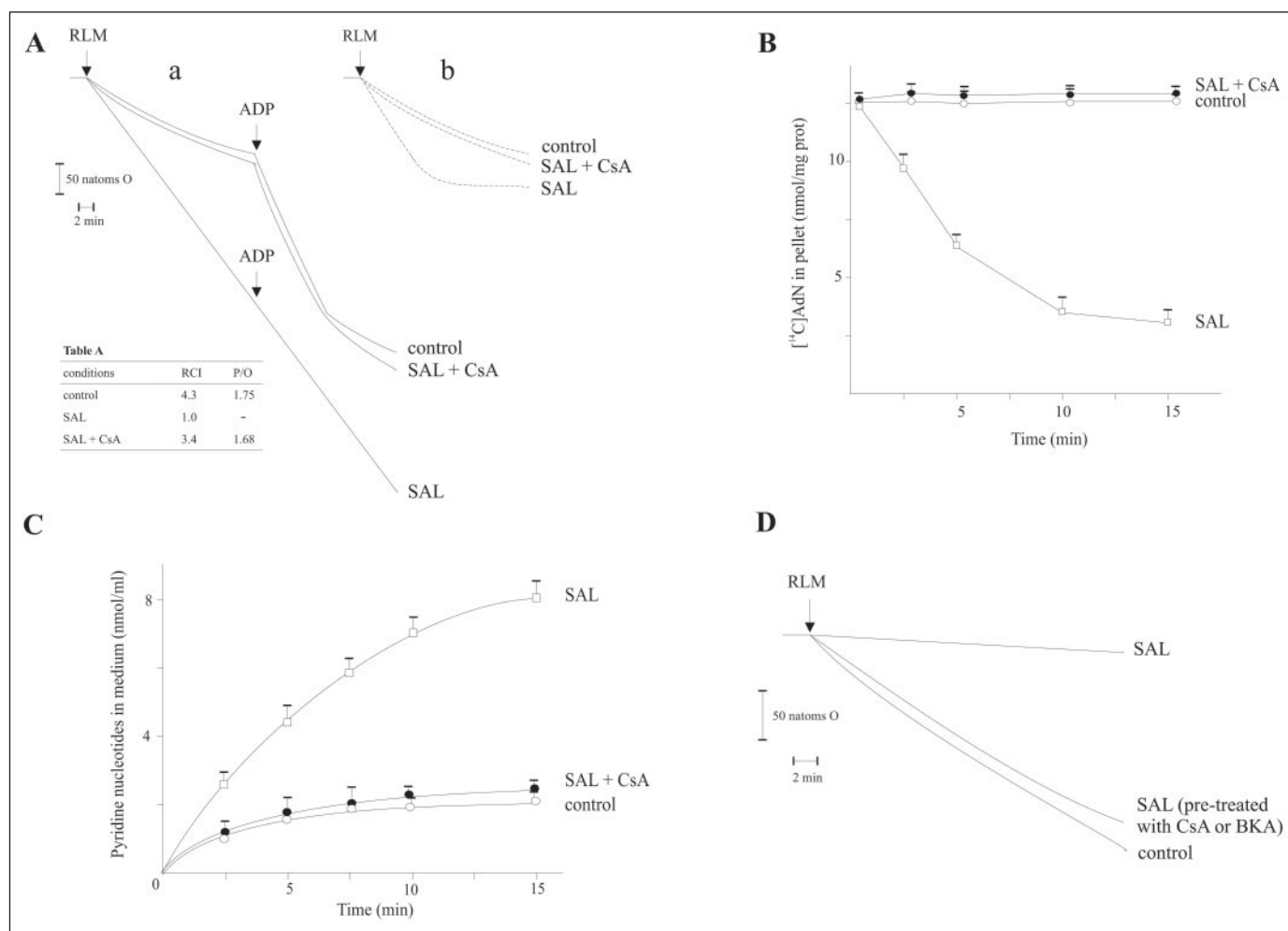


FIGURE 2. Effect of salicylate on succinate and β -hydroxybutyrate oxidation and oxidative phosphorylation (A), adenine (B) and pyridine (C) nucleotide mitochondrial content, and palmitoylcarnitine oxidation (D). A–C, RLM were incubated in standard medium as described under “Experimental Procedures,” in the presence of 1 mM phosphate. Salicylate (SAL) and CsA were present at 0.5 mM and 1 μ M, respectively. Dashed lines in A, b, presence of 5 mM β -hydroxybutyrate as substrate instead of succinate plus rotenone. Where indicated in A, a, additions of 0.2 mM ADP for respiratory control index and phosphate to oxygen ratio calculations. Adenine nucleotides (AdN) (B) and pyridine nucleotides (C) were measured in mitochondrial pellet and supernatant, respectively. D, RLM preincubated for 10 min as reported for A–C and treated as described under “Experimental Procedures,” were incubated in assay medium for β -oxidation, in presence of 30 μ M palmitoylcarnitine as substrate.

cyclosporin A (CsA) and bongkreic acid (BKA), typical inhibitors of MPT, confirming that the observed alterations are the results of pore opening (Figs. 1 and 2) (BKA was not used in the experiments shown in Fig. 2, A, B, and D, as it inhibits the translocase of adenine nucleotides).

The reducing agent DTT, the alkylating NEM, and the antioxidants butylhydroxytoluene and Trolox also inhibit swelling and $\Delta\Psi$ collapse (Fig. 3, A and B), indicating that oxidative stress is involved in the phenomenon as inducer, amplifier, or both. However, although the results of Fig. 3 are indicative of the onset of oxidative stress, catalase and mannitol, well known scavengers of ROS, are ineffective in protecting RLM against MPT (results not reported). The inability of catalase, and most probably also of mannitol, may be consistent with the sidedness of ROS generation within mitochondria.

In the absence of Ca^{2+} , salicylate causes a considerable increase in mitochondrial oxygen uptake when compared with controls (Fig. 4). Ca^{2+} , which alone has only a negligible effect, causes a further increase in oxygen uptake (Fig. 4). These observations, particularly notable in the absence of Ca^{2+} , mean that salicylate, alone, can cause the generation of hydrogen peroxide or other ROS. Fig. 5A shows that the measurement of H_2O_2 production by mitochondria operating in a resting state (see controls in the absence or presence of Ca^{2+} , ~ 0.2 nmol/mg of protein), greatly increases up to 1.5 nmol/mg of protein in the presence of salicylate but in the absence

of Ca^{2+} . With Ca^{2+} , the measurement of H_2O_2 generation by salicylate further increases to 2.2 nmol/ of protein (Fig. 5B). CsA or BKA is ineffective in inducing inhibition when Ca^{2+} is absent (Fig. 5A); instead, in the presence of Ca^{2+} , both the inhibitors reduce the level of H_2O_2 generation to the same value as in the absence of the cation (Fig. 5B). The real amounts of H_2O_2 generated by salicylate are most probably higher than those reported, because, as soon it is generated, H_2O_2 reacts with its targets and may also be transformed into other ROS (see also the description of the assay under “Experimental Procedures”). The production of hydrogen peroxide and, most probably of other ROS, explains the onset of oxidative stress, the effects of which are shown in Fig. 6. This figure shows that, without Ca^{2+} , salicylate induces a drop of $\sim 40\%$ in the total contents of reduced thiol groups and of 55% with the cation (Fig. 6A). However, Ca^{2+} alone induces a drop in reduced thiols of $\sim 15\%$. NEM almost completely prevents the oxidizing effect of salicylate.

Salicylate can also oxidize glutathione in the absence and presence of Ca^{2+} by ~ 20 and 30%, respectively, whereas Ca^{2+} alone causes oxidation of 17–18% (Fig. 5B). Also in this case, NEM strongly inhibits glutathione oxidation in both conditions.

Besides sulfhydryl groups and glutathione, another target of pro-oxidants agents in mitochondria are pyridine nucleotides. Fig. 7 shows that salicylate can induce strong pyridine nucleotide oxidation, but this

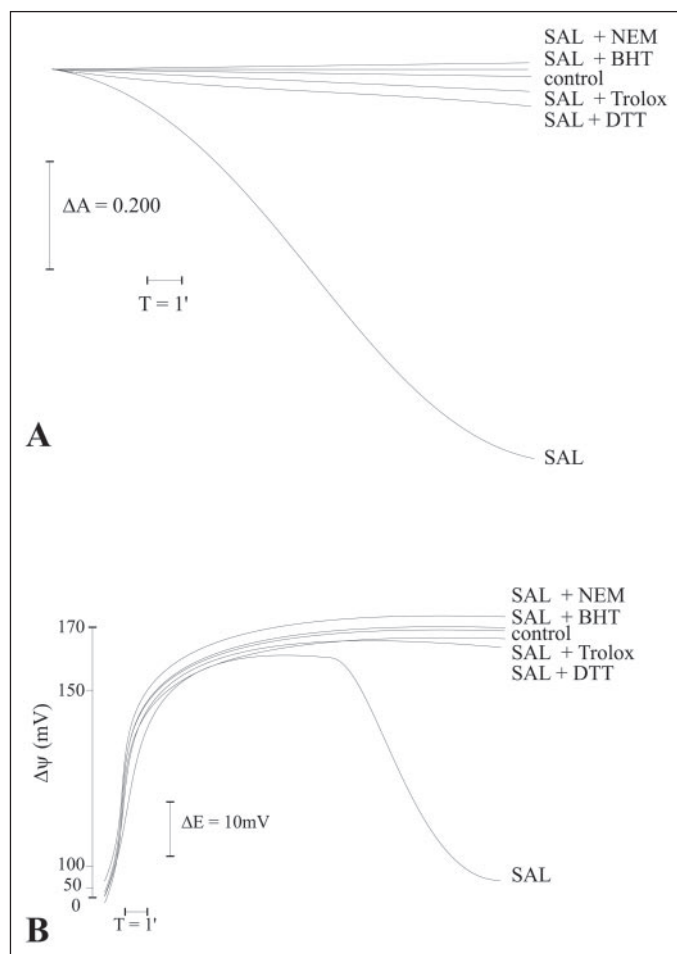


FIGURE 3. Effect of antioxidantizing agents on mitochondrial swelling (A) and $\Delta\Psi$ collapse (B) induced by salicylate. Incubation conditions and salicylate concentration as in the legend to Fig. 1. When added to medium, 1 mM DTT, 10 μ M NEM, 25 μ M butylhydroxytoluene, and 100 μ M Trolox were present. Both assays were performed four times, with comparable results.

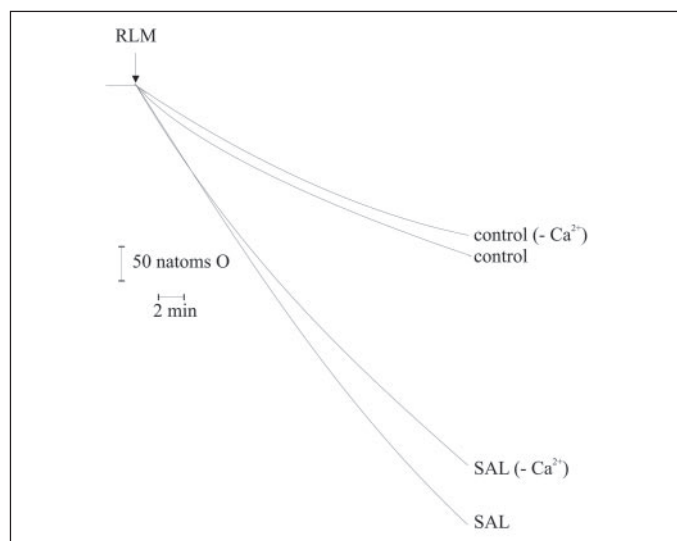


FIGURE 4. Increased oxygen uptake induced by salicylate. Incubation conditions and salicylate (SAL) concentration as in the legend to Fig. 1. Where indicated, standard medium was deprived of Ca^{2+} . Data are representative of four similar experiments.

effect is observable only in the presence of Ca^{2+} ; in its absence, no oxidation is observable, and Ca^{2+} alone is ineffective. NEM and DTT prevent oxidation. Oxidation of pyridine nucleotides probably occurs

after pore opening, as they are not oxidized in the absence of Ca^{2+} . It is noteworthy that oxidized pyridine nucleotides are released from mitochondria (see Fig. 2C). This accounts for the results in Fig. 2D, in which palmitoylcarnitine oxidation is blocked in RLM undergoing MPT induction.

It is generally agreed that MPT is the first step of the proapoptotic pathway. This characteristic is because of the release of soluble proapoptotic factors from mitochondria following massive swelling and consequent outer membrane rupture. Western blot analyses (Fig. 8) show that, in the presence of Ca^{2+} , salicylate induces the efflux of Cyt c (Fig. 8A) and AIF (B) from RLM. In the absence of Ca^{2+} , no efflux is observable. CsA and NEM are able to prevent both releases.

Comparisons between the effects of salicylate and acetylsalicylate in inducing MPT are shown in Fig. 9 and highlight the greater efficacy of salicylate in causing mitochondrial swelling (Fig. 9). Instead, benzoate is completely ineffective. The reduced effect of acetylsalicylate with respect to salicylate and the complete inefficacy of benzoate were also observed when measuring thiol, glutathione, and pyridine nucleotide oxidation and in the release of proapoptotic factors (results not reported). As acetylsalicylate is known to hydrolyze to salicylate in the cellular environment, the reduced efficacy observed in the experiment of Fig. 9 is because of incomplete hydrolysis of acetylsalicylate by mitochondrial esterases. The complete inefficacy of benzoate means that the reactive center of salicylate is the hydroxyl group.

For information about the site(s) of action of salicylate on the mitochondrial membrane, we considered the possibility of its interaction with some transition metal of the respiratory chain, in the oxidized state, using the same approach used for flavonoids (29) and triterpenoids (41). The basis of this strategy was to induce various redox levels in the respiratory complexes, to identify their involvement in the interaction. Fig. 10 shows that RLM incubated with salicylate in the presence of β -hydroxybutyrate as energizing substrate, which gives electrons to Complex I, exhibits considerable swelling (Fig. 10A), accompanied by rapid and complete $\Delta\Psi$ collapse (B). The addition to the medium of the inhibitor rotenone, which blocks β -hydroxybutyrate oxidation, and succinate, which restores electron flux at the level of Complex II, produces almost complete inhibition of the mitochondrial swelling induced by salicylate (Fig. 10C), whereas $\Delta\Psi$ undergoes only a slight transient drop (D).

When RLM are energized with ascorbate plus trimethyl-*para*-phenyldiamine (which gives electrons to Cyt c) and are again incubated in the presence of β -hydroxybutyrate plus rotenone and succinate plus antimycin A (which maintain reduced Complexes II and III) (42), the inhibition of swelling is slightly less marked than in the previous experiment and is accompanied by a small $\Delta\Psi$ drop (results not reported).

When β -hydroxybutyrate acts as an energizing substrate, the production of H_2O_2 by salicylate, determined in the absence of Ca^{2+} , increases by ~ 0.5 nmol/mg of protein when compared with that normally obtained when the substrate is succinate plus rotenone (Fig. 5A). Instead, the presence of β -hydroxybutyrate, when used as a reducing agent of Complex I, together with succinate plus rotenone, almost completely inhibits H_2O_2 generation (Fig. 11).

The variable amounts of H_2O_2 produced by salicylate in the various experimental conditions shown in Fig. 11 exactly reflect its effect at the level of MPT induction shown in Fig. 10. In the presence of Ca^{2+} , H_2O_2 production is increased when β -hydroxybutyrate is the energizing substrate (results not reported).

DISCUSSION

The results reported here demonstrate that the well known induction of MPT by salicylate (see Figs. 1 and 2) is the result of oxidative stress

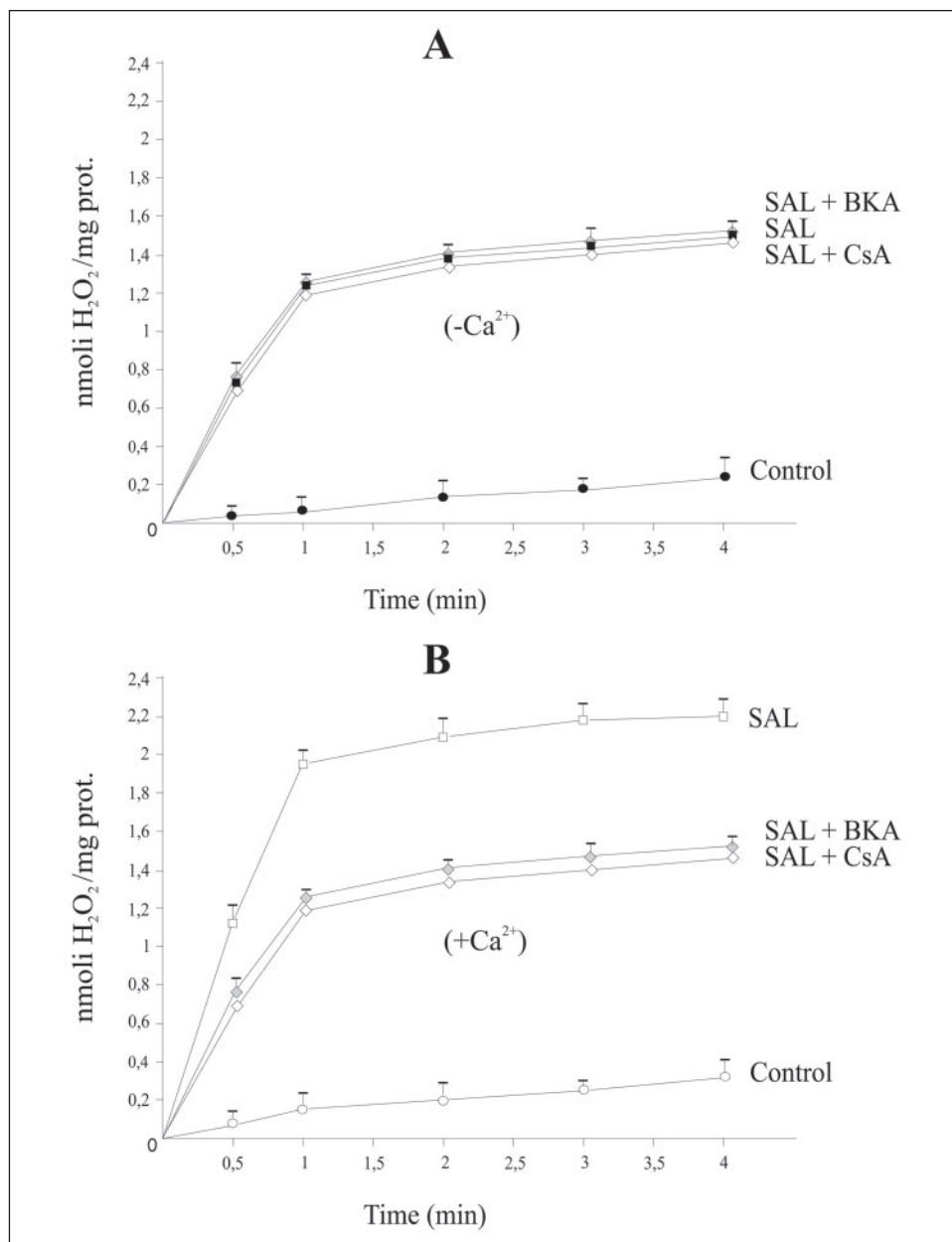


FIGURE 5. Hydrogen peroxide generation by mitochondria induced by salicylate in absence (A) or presence (B) of Ca²⁺. Incubation conditions and salicylate (SAL) concentration as in Fig. 1. Where indicated, 1 μ M CsA and 5 μ M BKA were present. Mean values + S.D. of four experiments are shown.

triggered by the interaction of salicylate with the respiratory chain in the presence of Ca²⁺. The first indication of the involvement of such a process is given here by the results shown in Fig. 3, *i.e.* that several reducing, antioxidizing, or alkylating agents (DTT, butylhydroxytoluene, Trolox, NEM) can inhibit the induction of MPT by salicylate. The significant increase in oxygen uptake caused by salicylate in the absence of Ca²⁺ (Fig. 4), correlated with the production of hydrogen peroxide in the same condition (Fig. 5A), is a clear demonstration of the pro-oxidant effect of salicylate. This action is further confirmed by the oxidation undergone by sulfhydryl groups and glutathione and the complete prevention exhibited by NEM (Fig. 6, A and B) when RLM are again subjected to salicylate in the absence of Ca²⁺. It should be noted that, in these conditions, although oxidative stress is established, the permeability transition is not triggered (Fig. 1, curves (SAL (-Ca²⁺))). The results reported in Fig. 5A showing that MPT inhibitors CsA and BKA do not induce any inhibition of H₂O₂ generation, unequivocally confirm that the observed oxidative stress is not because of pore opening. This phe-

nomenon takes place only if Ca²⁺ is also present (Fig. 1). The increase in oxygen uptake (Fig. 4), H₂O₂ generation (Fig. 5B), and enhanced oxidation of thiol groups, glutathione (Fig. 6), and pyridine nucleotides (Fig. 7), observed in the presence of Ca²⁺, are the result of the opening of the transition pore. That is, with pore opening, the electrochemical gradient collapses, and the corresponding increase in electron flux along the respiratory chain to restore it results in an increase in oxygen uptake and, consequently, of ROS production by the chain. This is responsible for the increase in thiol and glutathione oxidation and oxidation of pyridine nucleotides. The maintenance of the reduced state, as controls shown by the presence of NEM (Figs. 6 and 7) and DTT (Fig. 7), is further confirmation of increased oxidative stress in the presence of Ca²⁺.

The observation that in this condition CsA and BKA diminish H₂O₂ generation to the same level as that seen in the absence of Ca²⁺, that is, they block H₂O₂ generation because of the opening of the pore, further confirms that MPT is responsible only for enhanced oxidation of thiols

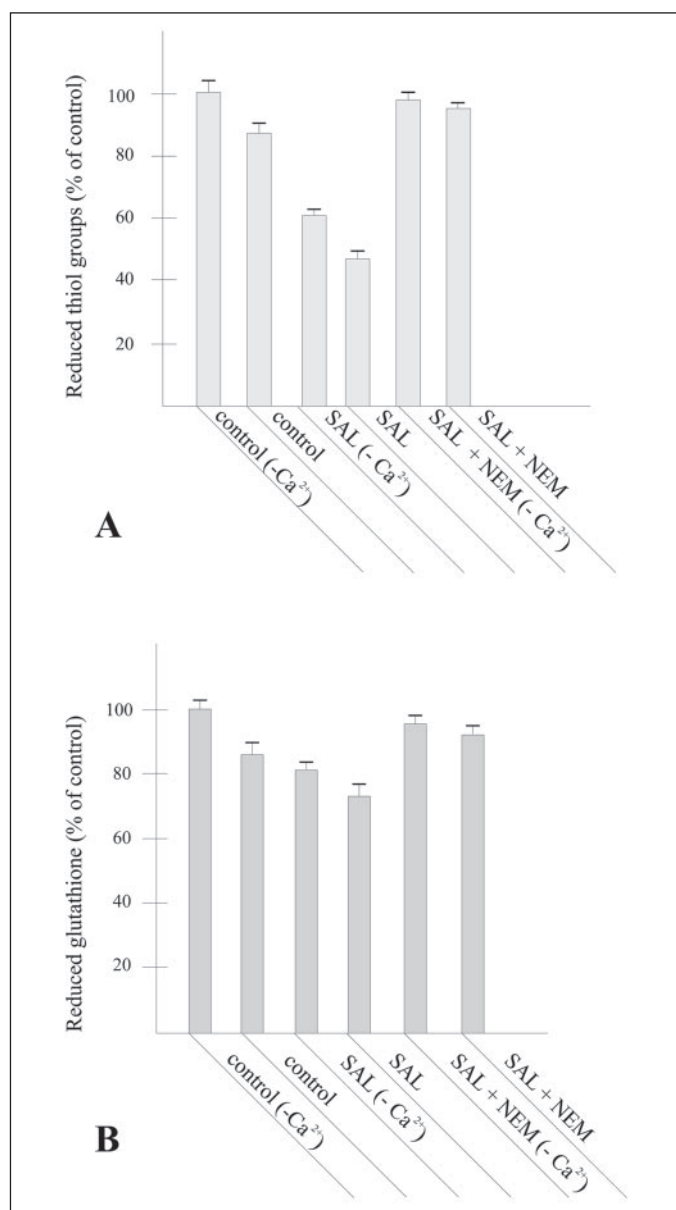


FIGURE 6. **Mitochondrial thiol (A) and glutathione (B) oxidation by salicylate.** Incubation conditions and salicylate (SAL) concentration as in Fig. 1. When present, NEM was 10 μM . Mean values + S.D. of three experiments are shown.

and glutathione and oxidation and efflux of pyridine nucleotides. In conclusion, the sequence of the events is ROS production by salicylate, MPT induction by ROS, and further ROS production by pore opening.

One proposable mechanism for pore opening is that H_2O_2 , or other ROS produced by salicylate, act at the level of critical thiol groups (43) located on adenine nucleotide translocase. This is generally known to be the main protein involved, the oxidation of which forms dithiols, and is responsible for pore opening in the presence of Ca^{2+} . The inefficacy in inhibiting MPT by catalase seems to demonstrate that H_2O_2 is not directly involved in the phenomenon. However, it must be noted that the enzyme cannot cross the membrane and that peroxide generation does not always rapidly diffuse out of the mitochondrion. Instead, glutathione oxidation (Fig. 6B), which demonstrates the involvement of the glutathione peroxidase/glutathione reductase system in the induction of the phenomenon, confirms that H_2O_2 is also an active agent (44). Fig. 7 clearly demonstrates that oxidation of pyridine nucleotides may be

considered as one effect of pore opening, rather than as a primary event for induction of the phenomenon, as no oxidation is observable in the absence of Ca^{2+} .

Ca^{2+} alone can also induce the oxidation of sulfhydryl groups and glutathione (Fig. 6), although without inducing MPT (Fig. 1). This effect is most probably because of the generation of hydrogen peroxide induced by the cation (see Fig. 5), like the results of its interaction with membrane cardiolipin. The change in membrane organization that follows this interaction may affect coenzyme Q mobility and favor ROS production (45). But the critical thiol groups responsible for pore opening are very probably not oxidized in this condition.

In considering MPT induction by salicylate as the result of oxidative stress, the generation of hydrogen peroxide probably follows the same pathway previously observed with the isoflavonoid genistein (29). Fig. 9 demonstrates that the reactive group of salicylate responsible for the observed effects is the hydroxyl group. As the reactive group of genistein and other flavonoids is also a hydroxyl group (41, 46), the generation of hydrogen peroxide may be explained by the reaction sequence reported in works in which a transition metal in the oxidized form, Fe^{3+} or Cu^{2+} , was involved in the first step (e.g. Ref. 46).

Fig. 10 shows that the maximum extent of MPT induction by salicylate, evaluated as mitochondrial swelling (A) and $\Delta\Psi$ collapse (B), is obtained when β -hydroxybutyrate is used as substrate, as the result of H_2O_2 generation. This effect is almost completely abolished by the addition of succinate plus rotenone (Fig. 10, C and D), which triggers the reduction of all the compounds of Complex I and prevents the production of H_2O_2 (Fig. 11). This indicates that the main and, most probably, the only target of salicylate is an Fe^{3+} of some Fe-S cluster of Complex I. That is, if some transition metal of all the other complexes were involved in the interaction with salicylate, the addition of succinate plus rotenone would not cause complete inhibition of MPT. Bearing in mind that the redox couple of the hydroxyl group of salicylate is of the ubiquinone/semiquinone type, the standard redox potential of which is about -60 mV (46), one hypothesis is that the interacting Fe^{3+} belongs to the N-2 (Fe-S) center of Complex I, as it has a redox potential in the range of -20 to -160 mV (47). A control experiment with ascorbate as the energizing substrate, in which Complexes I, II, and III were in a completely reduced state (results not reported), only demonstrates the induction of MPT to a very low extent. This effect was most probably because of the presence of antimycin (48) and may be considered negligible.

As discussed above, the hypothesized target of the reactive group of salicylate is the N-2 center of Complex I. This indicates that the compounds which react by means of a redox couple of ubiquinone/semiquinone type interact with the respiratory chain at the level of the last part of Complex I or bc_1 Complex. Genistein has also been proposed to interact with b_H heme (28) and the cyclic triterpene, glycyrrhetic acid, with the N-2 center (41).

In conclusion, the results reported here highlight important data regarding the interaction mechanism of salicylates with the mitochondrial membrane responsible for induction of MPT. One of the most important functions of salicylates is their well known anti-inflammatory and antioxidant capacity, exhibited on a physiological level. Thus, the main result of this study, i.e. induction of MPT by oxidative stress, apparently seems to be a contradiction. However, it should be borne in mind that other drugs such as flavonoids (46), isoflavonoids (29), cyclic terpenoids (41), and cinnamyl ketones (49), which normally behave as ROS scavengers, in particular conditions may also exert pro-oxidizing activity, as observed with salicylate. This possibility is due, as mentioned above, to the characteristic of the reactive redox couple of these com-

FIGURE 7. Endogenous pyridine nucleotide oxidation by salicylate. Inhibition by antioxidantizing agents. RLM incubated as in Fig. 1. Salicylate (SAL) present at 0.5 mM concentration. When added to medium, 10 μ M NEM and 1 mM DTT were present. Where indicated, standard medium was deprived of Ca^{2+} . Three additional experiments gave comparable results.

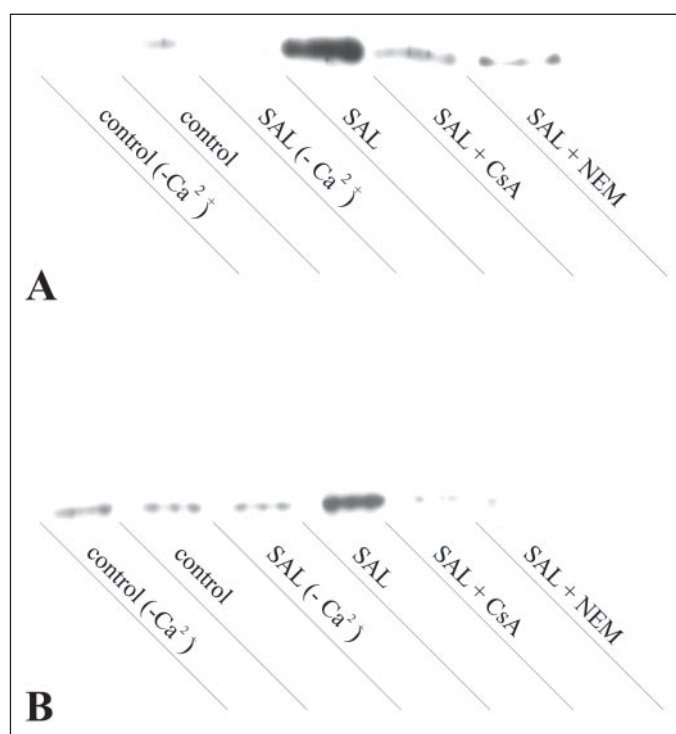
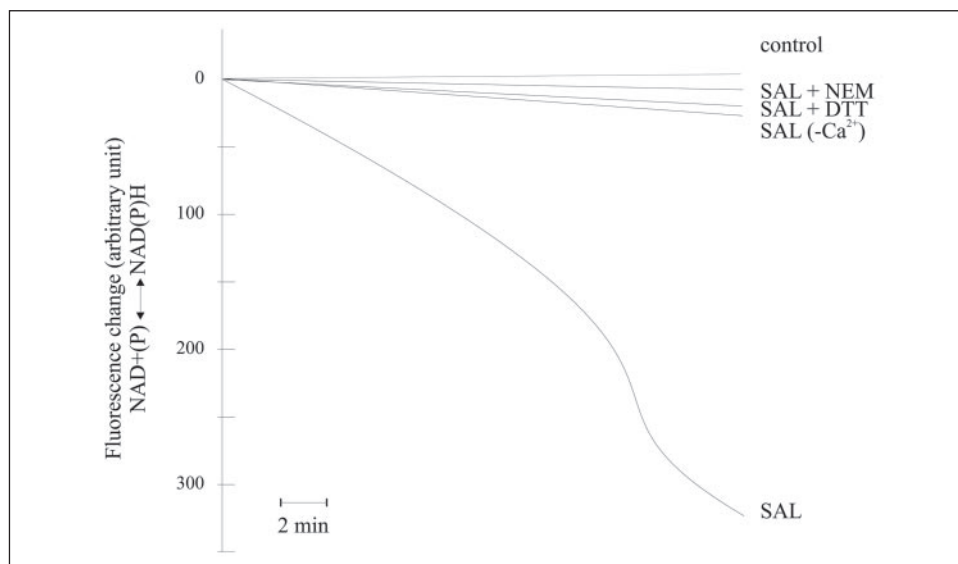


FIGURE 8. Release of cytochrome *c* (A) and AIF (B) induced by salicylate. Inhibitory effect by CsA and NEM. RLM incubated for 15 min in standard medium and, where indicated, deprived of Ca^{2+} . Salicylate (SAL) at 0.5 mM concentration. When added to medium, 1 μ M CSA and 10 μ M NEM was present. Data are representative of three separate experiments.

pounds to interact with particular transition metals of the respiratory chain.

The close correlation between oxidative stress, MPT, and apoptosis, as also confirmed by the release of cytochrome *c* and AIF, inhibited by NEM and CsA (Fig. 8), indicates that salicylate is a pro-apoptotic agent and, in particular conditions, has an antineoplastic action. In this regard, it has been observed that salicylate is able to cause apoptosis in cultured hepatocytes (12). Indeed, aspirin and other non-steroid anti-inflammatory compounds exert their chemopreventive action against cancer by inducing apoptosis in transforming cells (50).

In the exhaustive study performed by Trost and Lemasters (39) it was

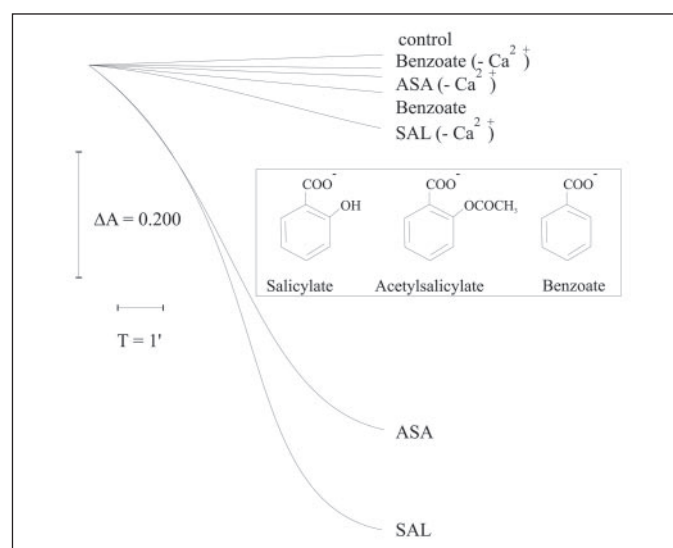


FIGURE 9. Mitochondrial swelling induced by salicylate, acetylsalicylate, and benzoate. RLM were incubated in standard medium, as described under "Experimental Procedures." Where indicated, medium was deprived of Ca^{2+} . Salicylate (SAL), acetylsalicylate (ASA), and benzoate present at 0.5 mM. Experiment was performed four times, with identical results.

emphasized that a cardinal feature of Reye's syndrome is the formation of microvesicular steatosis. This is a typical pathological event in the liver, in which small fatty droplets accumulate in the hepatocytic cytosol without displacing the control nucleus. The above authors also point out that salicylate causes Reye's syndrome by inhibiting mitochondrial energy production and inducing mitochondrial damage. The results reported here, besides giving a general picture regarding the induction of MPT by salicylate and the mechanism of its action, may also provide further elucidations regarding the involvement of MPT in Reye's syndrome. The main goal of our study was to demonstrate that salicylate induces MPT by means of oxidative stress in the presence of Ca^{2+} . However, it is noteworthy that the opening of the transition pore provokes further oxidative stress leading to increases in thiol and glutathione oxidation. Indeed, in this condition, pyridine nucleotides are also oxidized (Fig. 7) and released from the mitochondria (Fig. 2C). This finding accounts for the observed block of NAD-dependent substrate oxidation (Fig. 2A, b) and consequently the suppression of the citric acid

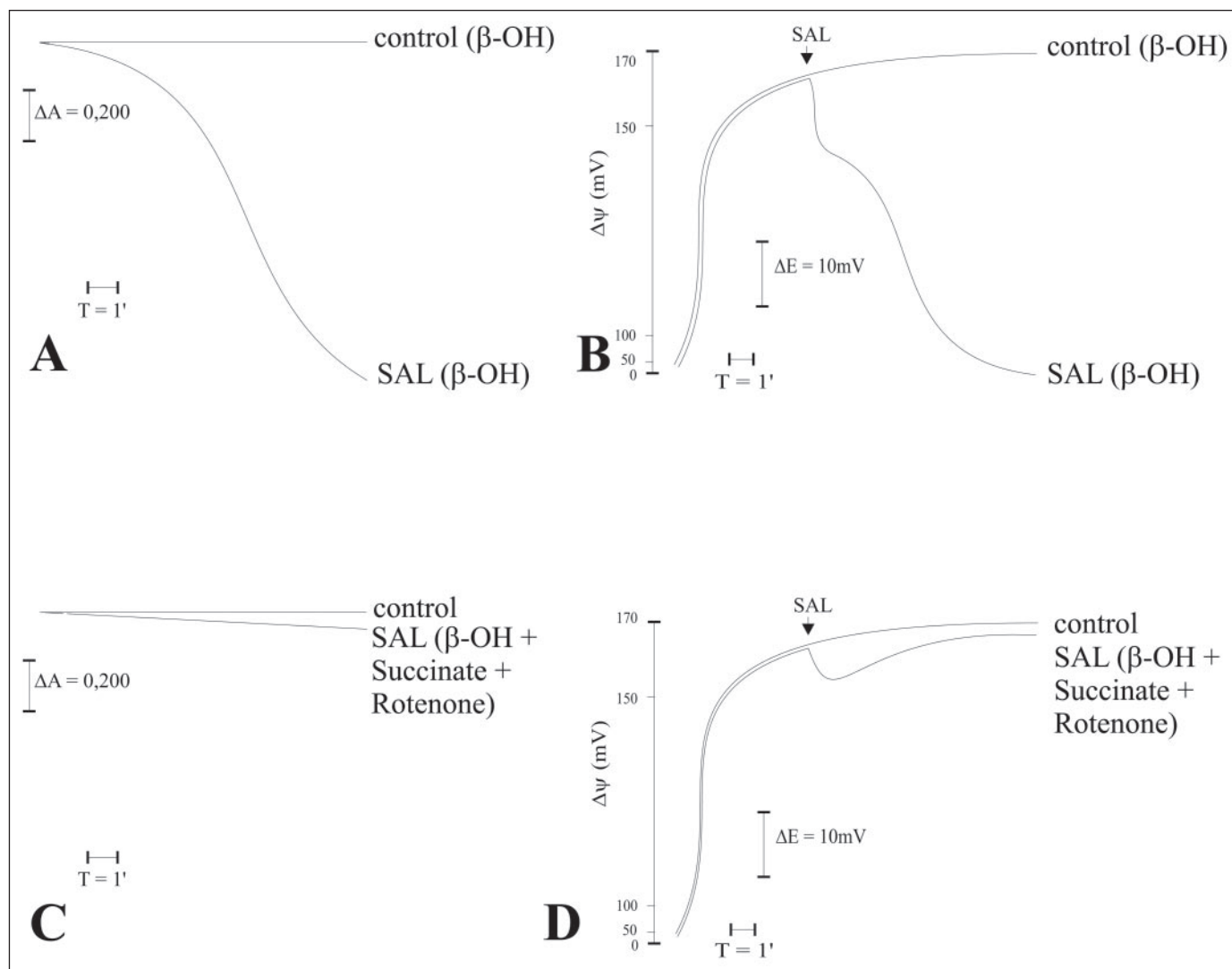
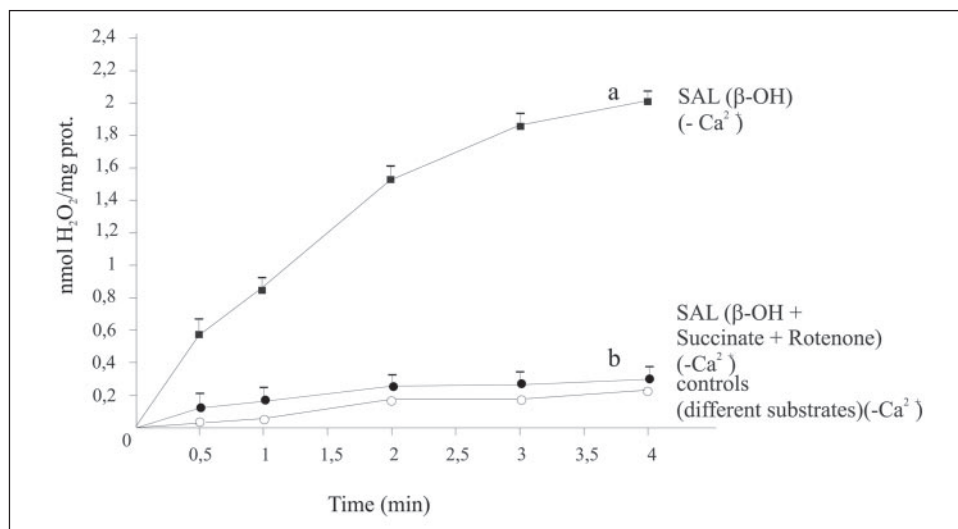


FIGURE 10. Effect of various redox states of respiratory chain complexes and differing membrane energizing substrates on mitochondrial swelling (A, C) and $\Delta\Psi$ collapse (B, D) induced by salicylate. All incubations carried out as described in incubation procedures in a medium containing 250 mM sucrose, 10 mM Hepes (pH 7.4), and $40\ \mu\text{M}\ \text{Ca}^{2+}$. When present, salicylate was at 0.5 mM concentration. A and B, 5 mM β -hydroxybutyrate (β -OH) as energizing substrate. C and D, 5 mM succinate (energizing substrate), 1.25 μM rotenone, 5 mM β -hydroxybutyrate. Three additional experiments gave comparable results.

FIGURE 11. Hydrogen peroxide generation by mitochondria treated with salicylate, in absence of Ca^{2+} , in differing redox states of respiratory chain complexes. General incubation conditions as in the legend to Fig. 10, except that medium was deprived of Ca^{2+} . Where indicated, medium was energized by β -hydroxybutyrate (β -OH) (trace a) or succinate plus rotenone in presence of β -hydroxybutyrate (trace b), at concentrations as shown in Fig. 9. Control traces refer to both energizing conditions.



cycle and gluconeogenesis, which are consistent with clinical pathology changes in Reye's syndrome (39). The depletion of oxidized pyridine nucleotides also explains the complete inhibition of palmitoylcarnitine oxidation (Fig. 2D) and, more generally, the suppression of β -oxidation. As a consequence of this impairment, there is an increased cytoplasmic level of fatty acids, with the generation of microvesicular steatosis. Other metabolic alterations in Reye's syndrome are consistent with mitochondrial uncoupling and suppression of ATP synthesis by oxidative phosphorylation. These events are consistent with the observed effect of MPT in the complete lowering of respiratory control index and phosphate to oxygen ratio (Fig. 2A).

As reported in the literature (39) and also confirmed by the results shown in Figs. 1 and 2, CsA and some its analogs may be considered as potential therapeutic agents against Reye's syndrome. However, other compounds with antioxidizing properties and able to inhibit MPT induction, e.g. propargylamines and indolalkylamines, used in Parkinson disease (51–53), may also exert effective therapeutic action in the treatment of Reye's syndrome. Very recently the compound MitoQ has been proposed as a potent antioxidant, as its mitochondrial transport is driven by $\Delta\Psi$, and it concentrates into the matrix several hundred-folds (54). This characteristic overcomes the limited efficacy of conventional antioxidants, because of the fact that they are difficult to deliver to mitochondria *in situ*. The use of targeting antioxidants to mitochondria "in vivo" may be a new therapeutic strategy for Reye's syndrome, as well as for other diseases.

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