Biochemical Pharmacology, Vol. 33, No. 18, pp. 2869–2876, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

REYE'S SYNDROME: SALICYLATES AND MITOCHONDRIAL FUNCTIONS

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(*Received* 3 *November* 1983; *accepted* 16 *January* 1984)

Abstract—The effects of aspirin (acetylsalicylate, ASA) and related compounds in the presence of Ca^{2+} on the oxidative metabolism of isolated rat liver mitochondria were studied. Intact mitochondrial preparations preincubated with ASA + Ca^{2+} exhibited a transient stimulation of the state 4 respiratory rate with NAD^+-linked substrates, followed by an inhibition which could not be released by the addition of ADP or uncoupler. Maximum respiratory rates were achieved by subsequent addition of NAD^+ or succinate. The Ca^{2+}-transport inhibitors ruthenium red and ethylene glycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) prevented these effects. Five brands of commercial aspirin were tested and were as effective as purified ASA. Tylenol (acetaminophen) could reproduce these effects only at much higher (≧ 10-fold) concentrations. Other salicyl derivatives showed results qualitatively similar to ASA, with potencies in the order: acid >>> ASA >> alcohol ≧ catechol > amide, salicylate being approximately 10-fold more potent than ASA. The magnitude of the effect seen depended on the Ca^{2+} (endogenous + exogenous) and salicylate concentrations/mg mitochondrial protein, and on the length of the preincubation. Added inorganic phosphate was also required. That salicylate + Ca^{2+} induces an increase in the permeability of the mitochondrial inner membrane was demonstrated by the observation that 90% of the intramitochondrial $NAD(P)^+$ was released into the surrounding medium upon preincubation of intact mitochondria with these agents. Salicylate + Ca^{2+} had virtually no effect on respiration with succinate (+ rotenone) as substrate at salicylate concentrations which markedly affected NAD^+-linked substrate oxidation. The presence of rotenone in the preincubation mixture prevented the damaging effects of salicylate + Ca^{2+} on the mitochondrial membrane, suggesting that the redox state of intramitochondrial pyridine nucleotides can modulate these effects. The results reported here are similar to those reported previously by our laboratory for the effects of Reye's plasma and allantoin + Ca^{2+}, and indicate that, like these agents, salicylate and salicyl compounds can potentiate the Ca^{2+}-induced damage to the mitochondrial inner membrane and may be another factor responsible for Reye's syndrome.

Liver and brain are the two most noticeably affected tissues in Reye's syndrome [1]. Ultrastructural examinations of these two tissues [2–4] have shown marked abnormalities in mitochondrial structure, and decreased levels of mitochondrial, but not cytosolic, enzymes have been observed by both histochemical and biochemical assays [5, 6]. It has further been shown that the ultrastructural and histochemical abnormalities in Reye's syndrome are fully recoverable, which indicates that the mitochondrial lesion is acquired and transient [2, 7]. This, together with the absence of inflammation and the abrupt onset of and recovery (or death) from this disease, have suggested the action of a toxin (or toxins) in Reye's syndrome [8].

Recent studies in our laboratory [9] have demonstrated that plasma from Reye's syndrome patients induced a respiratory inhibition of isolated rat liver mitochondria, oxidizing NAD^+-linked substrates, which was due to an increased permeability of the mitochondrial membrane and the depletion of intramitochondrial NAD^+. This effect could be reproduced by a minute amount of allantoin (10^{-11} moles/mg mitochondrial protein) in combination with Ca^{2+} in the concentration range of normal plasma. Note: allantoin is the oxidation product of uric acid. These results strongly suggest that Reye's plasma contains a component(s) in critical concentrations which potentiate the effects of Ca^{2+} on mitochondrial function and that allantoin + Ca^{2+} may be the key components responsible for the mitochondrial disorder in Reye's syndrome.

The possible relationship of salicylate to Reye's syndrome has been the subject of much debate in recent years. The clinical symptoms of Reye's syndrome and salicylate poisoning can be strikingly similar, often making it difficult to distinguish between the two [8, 10]. Many Reye's syndrome patients have been reported to have received aspirin and were found to have measurable levels of salicylate in their blood [8, 10, 11]. In addition, the studies of Starko *et al.* [8], in which the consumption of various medications was compared, showed that the Reye's syndrome patients had consumed more salicylate-containing medications than the control children and that the severity of the disease appeared to be directly related to increasing doses of salicylate. In most cases of Reye's syndrome, however, the serum salicylate levels are not in the toxic range [10–12]. This has been one of the major arguments against a role for salicylate in Reye's syndrome.

On the other hand, it has long been recognized that salicylate and acetylsalicylate can act as uncouplers of oxidative phosphorylation [13–17], and they have been shown to inhibit a wide variety of cellular enzymes, *in vitro*, such as dehydrogenases and decarboxylases [17]. Uncoupling and the inhibition of mitochondrial dehydrogenases by salicylate are con-

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sistent with a role for this drug in the mitochondrial injury seen in Reye's syndrome.

In view of the controversy concerning the possible role of salicylates in Reye's syndrome, we have initiated a systematic study of the effects of aspirin and other salicyl derivatives in the presence and absence of Ca^{2+} on the respiratory functions of isolated rat liver mitochondria. Our results indicate that salicylates in combination with Ca^{2+} exert effects on the utilization of NAD^+-linked substrates which are virtually identical to those seen previously with Reye's plasma and allantoin + Ca^{2+}, and suggest that salicylates may be another possible agent involved in the mitochondrial lesion of Reye's syndrome. Part of the results has been communicated briefly [18, *].

MATERIALS AND METHODS

Mitochondria from the livers of male Sprague–Dawley rats which had been fasted for 16–24 hr were isolated by a modification of the method of Johnson and Lardy [19] as previously described [20].

Substrate oxidation rates and ADP/O ratios were determined polarographically [21] using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) fitted into a thermostatted plexiglass chamber with a capacity of either 1.0 or 3.0 ml. The reaction mixture consisted of 150 mM sucrose, 25 mM Tris–HCl, 10 mM phosphate buffer, pH 7.5, and 1.0 to 1.5 mg mitochondrial protein per ml. Additions were as indicated in the figures. Final volume was either 1.0 or 3.0 ml, as indicated in the figures; temperature = 30°.

Incubation conditions for monitoring the release of pyridine nucleotides from mitochondria were identical to those used in the polarographic studies, and have been described previously [9]. Extraction and fluorometric assay of the pyridine nucleotides were according to Williamson and Corkey [22] using a Perkin–Elmer MPF-2A fluorescence spectrophotometer (The Perkin–Elmer Corp., Norwalk, CT). The excitation wavelength was adjusted from 340 to 360 nm to reduce the background fluorescence due to salicylate.

The total calcium content of isolated rat liver mitochondria was assayed in perchloric acid extracts neutralized with K_2CO_3 [23]. The assays were performed using a Varian model 475 atomic absorption spectrophotometer (Varian Instrument Group, Palo Alto, CA).

The protein content was determined by the method of Lowry *et al.* [24] using crystalline bovine serum albumin as standard.

Reagents

Six brands of commercial analgesic tablets were purchased "over-the-counter" at a local drug store. These were: Perry Aspirin (Perry Drug Stores, Pontiac, MI), Bayer Aspirin (Bayer/Glenbrook Laboratories, NY), Bayer Children's Aspirin (Bayer/Glenbrook Laboratories), Empirin Aspirin (Burroughs Wellcome Co., NC), Bufferin (Bristol-Myers Co., NY), and Tylenol (McNeilab, Inc., PA). The analgesics were prepared by grinding several tablets and suspending the equivalent of 325 mg of the active ingredient (acetylsalicylic acid, ASA,* or acetaminophen), as determined by the average tablet weight, in 20 ml of 80% (v/v) dimethyl sulfoxide (DMSO); the final concentration of ASA in each sample was 90 mM (acetaminophen was 108 mM in the case of Tylenol tablets). The pH of each was adjusted to 7.4. The whole suspension (soluble + insoluble material) was used in the assays. Alternatively, the soluble and insoluble components were separated by centrifugation prior to use. Purified ASA, salicylate, or the other salicyl derivatives were dissolved in 80% DMSO and the pH adjusted to 7.4.

Reye's plasma was obtained from a patient in Stage 1 encephalopathy and was provided by Dr. E. Arcinue, Children's Hospital of Michigan. Normal plasma samples were obtained from healthy volunteers. Plasma samples were stored and prepared for assay as described previously [9].

All other chemicals of the purest grades available were obtained commercially. Glass redistilled water was used throughout the present investigation.

RESULTS

Effect of acetylsalicylate +Ca^{2+} on the respiratory activities of rat liver mitochondria utilizing β-hydroxybutyrate

Figure 1 shows typical polarographic traces of isolated rat liver mitochondria utilizing β-hydroxybutyrate as the respiratory substrate under various conditions. Untreated mitochondria (trace A) and those preincubated with 20 μM Ca^{2+} alone (trace B) both exhibited respiratory properties typical of tightly-coupled mitochondria. On the other hand, as can be seen in traces D and E, the respiratory properties of mitochondria which had been preincubated with acetylsalicylate [180 or 450 μM in 0.4% (v/v) DMSO] + 20 μM Ca^{2+} were altered such that the state 4 rate was initially stimulated and then inhibited. This inhibition could not be released by the subsequent addition of ADP or uncoupler (FCCP) (not shown). Maximum rates, however, could be achieved by the addition of either NAD^+ or succinate. These results are essentially as seen previously with Reye's plasma and allantoin + Ca^{2+} (cf. Fig. 1C and Ref. 9), indicating that the inhibition resulted from the depletion of intramitochondrial NAD^+ rather than from inhibition at the level of the respiratory chain or the phosphorylating enzymes. The NAD^+-stimulated rate retained rotenone sensitivity. As was seen with Ca^{2+} alone, neither DMSO alone (0.4%, v/v) nor DMSO + Ca^{2+} had any significant

* C. P. Lee, presented at the Workshop on Disease Mechanisms and Prospects for Prevention of Reye's Syndrome, National Institutes of Health, September, 1982.

* Abbreviations: ASA, acetylsalicylate; βOHB, β-hydroxybutyrate; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; RCI, respiratory control index; and RS-PL, Reye's plasma.

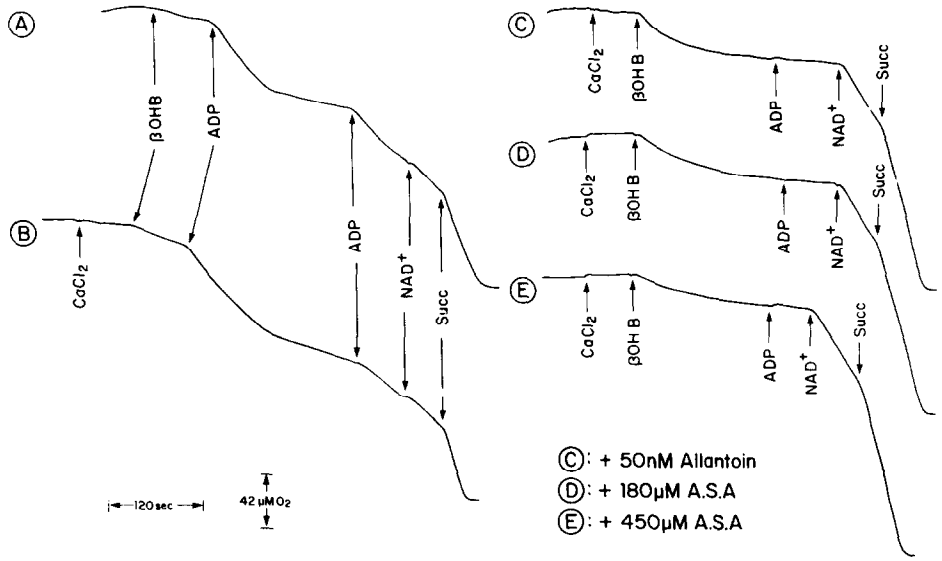

Fig. 1. Effects of ASA + Ca^{2+} on isolated rat liver mitochondria oxidizing β-hydroxybutyrate. Experimental conditions were as described in Materials and Methods. Other additions, where indicated, were 10 mM β-hydroxybutyrate, 300 μM ADP, 100 μM NAD^+, 5 mM succinate, 20 μM $CaCl_2$, and 1.5 mg mitochondrial protein/ml. Final volume = 1.0 ml. Total calcium (endogenous + added) = 20 nmoles/mg protein; preincubation time with ASA = 270 sec.

effect on respiration under identical incubation conditions. Similar results were also seen with other NAD^+-linked substrates such as pyruvate + malate (data not shown).

These results demonstrate that ASA + Ca^{2+} increased the permeability of the mitochondrial inner membrane and did so in a manner qualitatively similar to that seen previously with allantoin + Ca^{2+} and Reye's plasma (cf. Fig. 1C and Ref. 9).

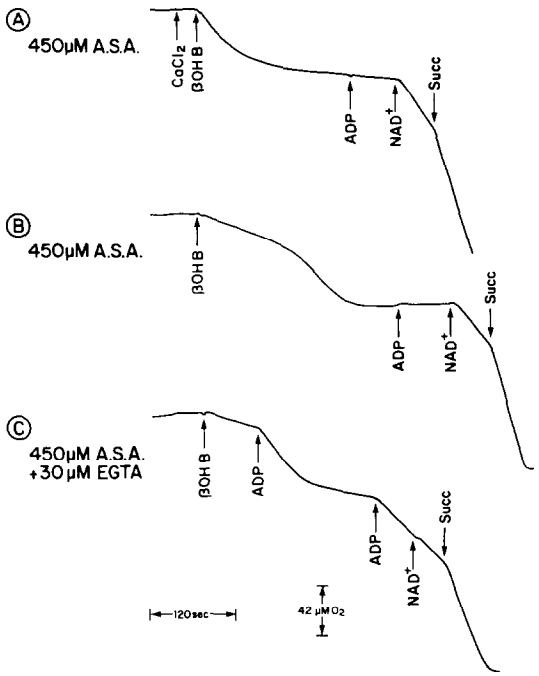

Fig. 2. Effect of EGTA. Experimental conditions were as described in the legend to Fig. 1, with the following exceptions: 1.3 mg mitochondrial protein/ml; total calcium = 21 nmoles/mg protein; preincubation time = 260 sec.

Calcium requirement

As in the cases of Reye's plasma and allantoin + Ca^{2+} [9], Ca^{2+} was required for the effects of ASA, as demonstrated in Fig. 2, which shows respiratory traces of β-hydroxybutyrate oxidation by rat liver mitochondria preincubated with ASA in the presence (trace A) and absence (trace B) of added Ca^{2+}, and in the presence of 30 μM EGTA (trace C). In the absence of added Ca^{2+}, the effect of ASA was diminished, and it was prevented completely by chelation of the endogenous calcium with EGTA. In addition, the effect of ASA + Ca^{2+} was prevented by the inclusion of ruthenium red (10 μM), a Ca^{2+} transport inhibitor, in the incubation mixture (not shown), indicating that Ca^{2+} transport played a vital role in the damage caused by the ASA + Ca^{2+}.

We also observed damage similar to that seen with ASA + Ca^{2+} when rat liver mitochondria were preincubated with Ca^{2+} alone at higher concentrations (> 30μM) or for longer incubation times (> 60 sec) than were routinely used in this study (data not shown). This indicates that the effect of ASA may have been an enhancement of the damaging effects of Ca^{2+} on the mitochondrial inner membrane. In the experiments reported here, the incubation conditions were controlled carefully to avoid any significant effect by Ca^{2+} alone (cf. Fig. 1B).

As described in detail below, salicylate, itself, was qualitatively similar to ASA in its effect on β-hydroxybutyrate oxidation and, like ASA, its effects were sensitive to both EGTA and ruthenium red. Salicylate was a considerably more potent mediator of the Ca^{2+}-induced membrane damage than ASA, however. Therefore, the further charac-

terization of the effects of these compounds on mitochondrial metabolism was done using salicylate rather than ASA.

Incubation conditions required for the effects of salicylates + Ca^{2+}

Both the occurrence and the magnitude of the effects on mitochondrial respiration induced by salicylates + Ca^{2+} were dependent on a number of variables. These are described below.

Salicylate concentration. The effect of salicylate concentration on the respiratory rates of rat liver mitochondria utilizing β-hydroxybutyrate is shown in Fig. 3. As can be seen, at concentrations below 10 nmoles/mg protein there was very little effect on the respiratory rates and virtually no stimulation of the rate upon addition of NAD^+. However, at higher concentrations of salicylate, the effects of salicylate paralleled the results depicted in Fig. 1, D and E. That is, the initial, state 4, rate was greatly stimulated (⊙—⊙) and then inhibited (△—△), and was not stimulated further by the subsequent addition of ADP (●—●). In addition, there was a significant stimulation of the rate by NAD^+ (○—○). Titration of the protein concentration while holding the concentration of salicylate constant demonstrated an inverse relationship between the amount of mitochondrial protein and the magnitude of the salicylate effect (data not shown), indicating that it was the salicylate/protein ratio rather than the absolute concentration that had to be considered. This result is, again, consistent with the previous results [9] which showed that the effect of Reye's plasma on isolated rat liver mitochondria depended on the ratio of Reye's plasma/mitochondrial protein.

Calcium concentration. A typical titration profile of the effects of salicylate as a function of the total calcium concentration (endogenous + exogenous) is shown in Fig. 4. The shaded area of the curve represents the total intramitochondrial calcium (free + bound), while the unshaded area represents added $CaCl_2$. This figure shows that the degree of effectiveness of salicylate + Ca^{2+} depended on the concentration of Ca^{2+} added to the incubation mixture as well

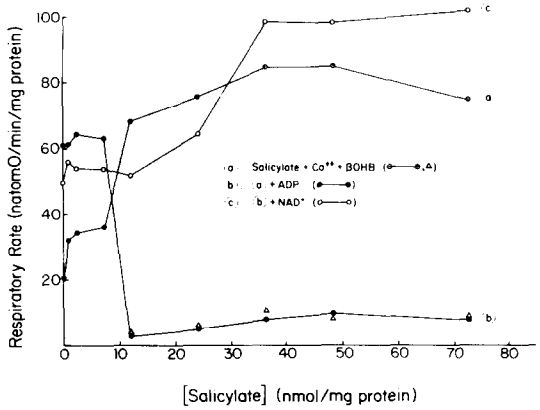

Fig. 3. Effect of salicylate concentration. Experimental conditions were as described in Fig. 1. Mitochondrial protein = 1.2 mg/ml; total calcium = 26 nmoles/mg protein; preincubation time = 210 sec. Final volume = 3.0 ml.

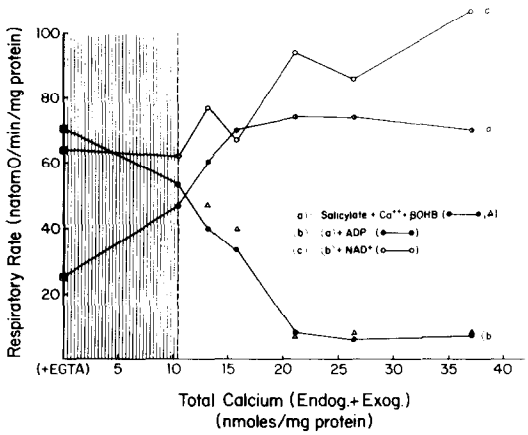

Fig. 4. Effect of calcium concentration. Experimental conditions were as described in Fig. 3. Mitochondrial protein = 1.2 mg/ml; salicylate = 300 μM; preincubation time = 240 sec. The shaded area represents the endogenous calcium content measured as described in Materials and Methods.

as on the content of endogenous calcium, although it is not known what proportion of this latter pool was actually participating in this process. The endogenous calcium contents of our mitochondrial preparations ranged from 5 to 20 nmoles/mg protein.

Incubation time. The effects of salicylates + Ca^{2+} also depended on the time of incubation with both salicylate and Ca^{2+} (data not shown). In both cases, a minimum incubation time was required for an effect on respiration to be seen. Increasing the length of preincubation with salicylate above this minimum increased the magnitude of the effect. There were severe constraints, however, on the maximum length of time that rat liver mitochondria could be preincubated with Ca^{2+} due to the damaging effects seen with Ca^{2+} alone (at 20–30 μM) at incubation times longer than 30–60 sec.

Phosphate concentration. In addition to the above variables the effect of salicylate + Ca^{2+} also required the presence of inorganic phosphate during the preincubation period. Elimination of phosphate from the incubation medium, or replacement of phosphate with acetate (data not shown), completely prevented the damaging effects of salicylate + Ca^{2+}. As demonstrated by the titration profile shown in Fig. 5, the minimum concentration of phosphate required was approximately 2 mM, with the maximum effect being seen at 10 mM or greater.

Fate of NAD^+

The data presented in Table 1 show the percentage of the total intramitochondrial NAD^+ and $NADP^+$ that appeared in the suspending medium under various incubation conditions. Approximately 90% of the pyridine nucleotide content was released upon incubation with salicylate + Ca^{2+}, whereas only about one-half was released by preincubation with either Ca^{2+} or salicylate alone. These results support our earlier conclusion that salicylate + Ca^{2+} increased the permeability of the mitochondrial inner membrane to these compounds.

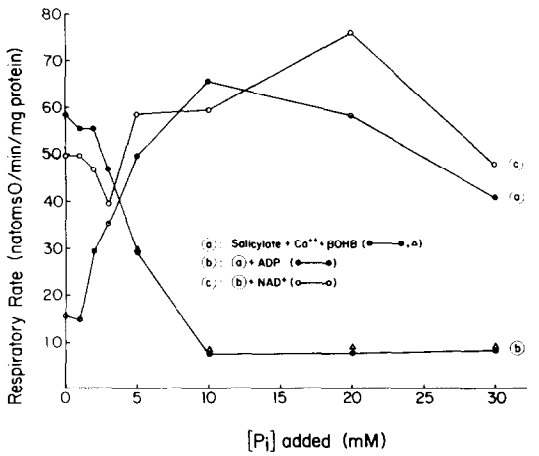

Fig. 5. Effect of phosphate concentration. Experimental conditions were as described in Fig. 3. Mitochondrial protein = 1.4 mg/ml; total calcium = 23 nmoles/mg protein; salicylate = 150 μM; preincubation time = 300 sec.

Table 1. Release of pyridine nucleotides from rat liver mitochondria following various treatments*

Additions	Percentage released	
	NAD^+	$NADP^+$
None	24 ± 5	38 ± 5
+ $CaCl_2$	39 ± 11	49 ± 4
+ Salicylate	54 ± 17	59 ± 13
+ Salicylate + $CaCl_2$	88 ± 3	92 ± 3

* Experimental conditions are as described in Materials and Methods. Other additions: 20 μM $CaCl_2$, 300 μM salicylate. Values are expressed as mean ± S.E. of four experiments. The total NAD^+ and $NADP^+$ contents were 2.3 ± 0.3 and 3.8 ± 0.6 nmoles/mg protein respectively.

Effects of salicylate + Ca^{2+} on succinate oxidation

Figure 6 shows the effects of salicylate + Ca^{2+} on the respiratory properties of mitochondria utilizing succinate (+ rotenone) as substrate. As can be seen from the polarographic traces in Fig. 6 and the titration curve in Fig. 7, 90 μM salicylate (+ Ca^{2+}), a concentration at which there is a significant effect on β-hydroxybutyrate oxidation (cf. Fig. 3), had virtually no effect on respiration with succinate as substrate. It was only at much higher concentrations ($\geq$ 900 μM) that an uncoupling effect of salicylate + Ca^{2+} was seen. This observation is consistent with reports in the literature that salicylate

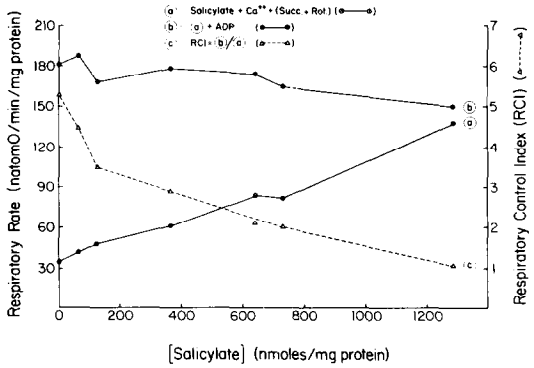

Fig. 7. Effect of salicylate concentration on the oxidation of succinate (+ rotenone). Experimental conditions were as described in the legend to Fig. 6.

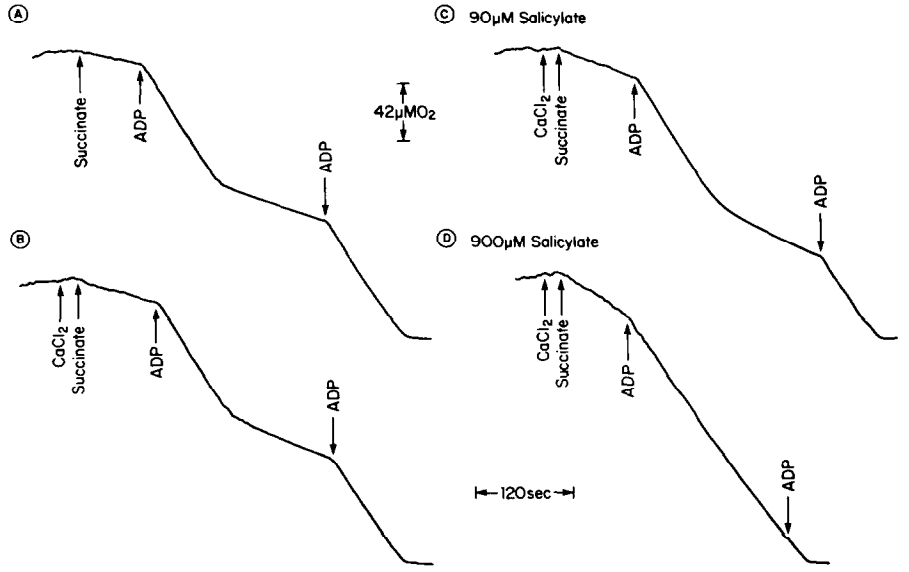

Fig. 6. Effect of salicylate + Ca^{2+} on rat liver mitochondria oxidizing succinate (+ rotenone). Experimental conditions were as described in Fig. 3. Rotenone = 1.7 μM; mitochondrial protein = 0.7 mg/ml; total calcium = 34.9 nmoles/mg protein; preincubation time = 135 sec.

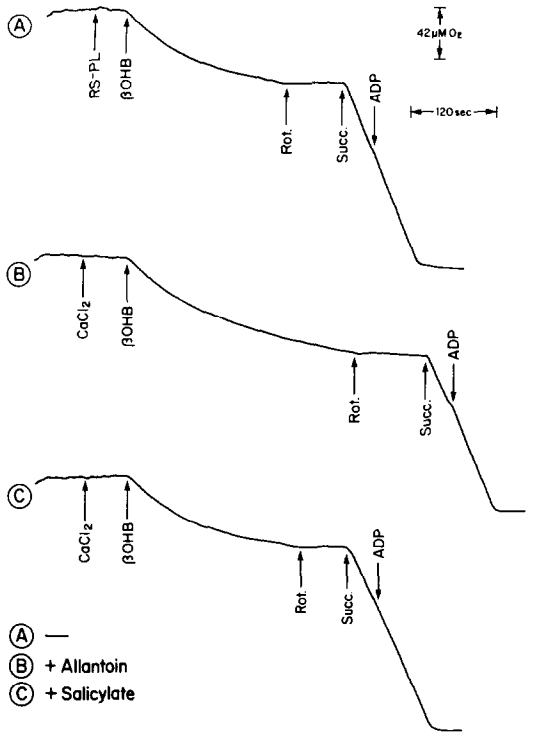

Fig. 8. Effect of preincubation with salicylate + Ca^{2+} in the absence of rotenone. Experimental conditions were as described in Fig. 3. Rotenone = 1.7 μM; mitochondrial protein = 1.1 mg/ml. Other conditions were: (A) Reye's plasma = 2.5 mg dry weight/ml, incubation time = 40 sec; (B) and (C) salicylate = 90 μM, allantoin = 100 nM; total calcium = 26 nmoles/mg protein; preincubation time = 300 sec.

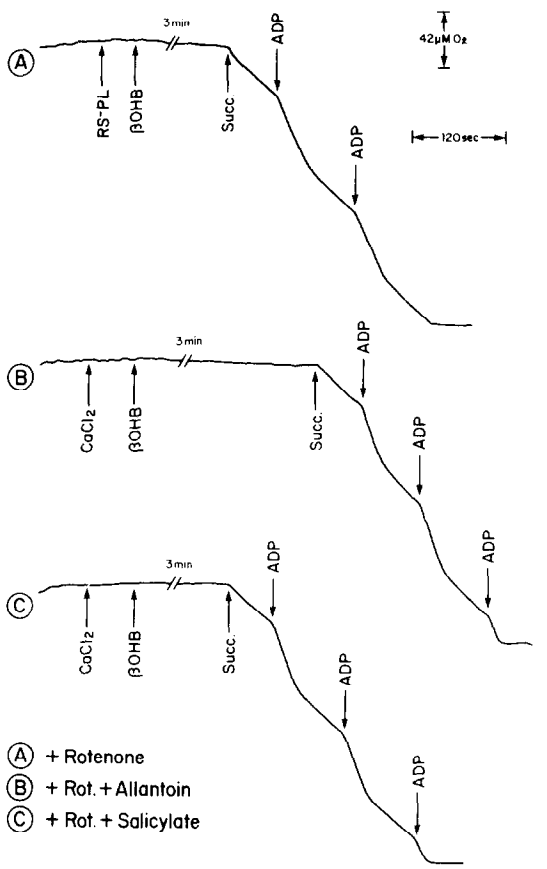

Fig. 9. Effect of preincubation with salicylate + Ca^{2+} in the presence of rotenone. Experimental conditions were as described in the legend to Fig. 8.

uncouples oxidative phosphorylation at concentrations greater than 0.5 mM [13–17]. However, it also indicates that there may be different mechanisms for the effects of salicylates + Ca^{2+} on the mitochondrial membrane in the presence of NAD^+-linked substrates or succinate (+ rotenone).

Role of the redox state of NAD(P)

The role of the redox state of the endogenous pyridine nucleotides in the action of salicylates + Ca^{2+} on the respiratory functions of rat liver mitochondria was examined. As shown in Fig. 8C, freshly isolated mitochondria were preincubated with salicylate + Ca^{2+} in the absence of rotenone. The effect of salicylate + Ca^{2+} on β-hydroxybutyrate oxidation was as described above (cf. Figs. 1 and 8), i.e. stimulation and then inhibition of the state 4 rate. Subsequent addition of rotenone followed by succinate resulted in stimulation of the respiratory rate, and this rate could not be stimulated further by the addition of ADP. On the other hand, when mitochondria were preincubated with salicylate + Ca^{2+} in the presence of rotenone (Fig. 9), β-hydroxybutyrate oxidation was inhibited completely, as expected, and subsequent addition of succinate, again, stimulated the respiratory rate. However, unlike the experiments in which rotenone was absent during the preincubation (Fig. 8), succinate oxidation retained ADP-induced respiratory control, indicating that the mitochondria remained functionally intact despite the preincubation with salicylate + Ca^{2+}. For comparison these experiments were also performed using mitochondria preincubated with Reye's plasma (Figs. 8A and 9A) and allantoin + Ca^{2+} (Figs. 8B and 9B). As can be seen, the results were virtually identical to those obtained with salicylate + Ca^{2+}. These data suggest that a high endogenous ratio of reduced/oxidized pyridine nucleotides (induced by the presence of rotenone and endogenous substrates) can protect mitochondria from the damaging effects of Ca^{2+} as potentiated by salicylate, allantoin or Reye's plasma.

Effects of commercial analgesics + Ca^{2+}

To determine whether commercial aspirin products contain ingredients besides ASA which also affect mitochondrial metabolism, rat liver mitochondria were preincubated, in the presence of 20 μM Ca^{2+}, with the five different brands of commercial aspirin tablets listed in Materials and Methods. The effects on β-hydroxybutyrate oxidation induced by these products were virtually identical to those of an equivalent concentration of purified ASA (cf. Fig. 1), both qualitatively and quantitatively. The DMSO-insoluble material by itself was without effect. These data indicate that ASA is the sole component respon-

sible for the effects seen and that the various additives (binders, fillers, buffer salts, flavorings, etc.), which may constitute as much as 60% of the tablet weight, neither enhance nor inhibit its effect. Tylenol tablets, which contain acetaminophen as the active ingredient, had much less effect on respiration and required a significantly higher concentration (approx. 10-fold) to approach in magnitude the effects seen with ASA or the commercial aspirin products.

Effects of other salicyl derivatives + Ca^{2+}

The roles of the salicylate moiety and the functional group in the action of ASA + Ca^{2+} on the mitochondrial inner membrane were assessed by preincubating rat liver mitochondria, in the presence of Ca^{2+}, with a series of salicyl derivatives, the structures of which are given in Fig. 10. The parent compound, salicylate, affected β-hydroxybutyrate oxidation in a manner qualitatively similar to that seen with ASA, but much more severely. Approximately one-tenth the amount of salicylate was required to produce the effect seen with ASA. The other salicyl derivatives tested were not as toxic to the mitochondria as either salicylate or ASA. The order of potency of the salicyl compounds was: salicylate >>> ASA >> salicyl alcohol ≥ catechol > salicylamide.

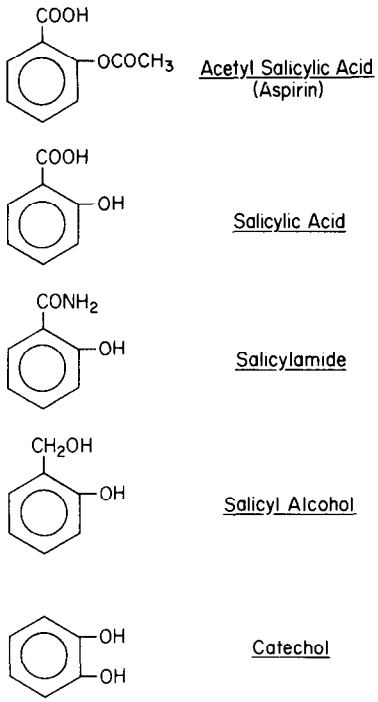

Fig. 10. Structures of various salicyl compounds.

DISCUSSION

It has been known for many years that Ca^{2+} and phosphate can cause an increase in the permeability of the mitochondrial inner membrane to Ca^{2+} which is accompanied by uncoupling (due to Ca^{2+} cycling) and inhibition of respiration with NAD^+-linked substrates [25–29]. The results reported here indicate that, like Reye's plasma and allantoin + Ca^{2+} (cf. Ref. 9), salicylates, at concentrations below those previously shown to uncouple oxidative phosphorylation [13–17], may act by potentiating the Ca^{2+}-induced increase in the permeability of the mitochondrial inner membrane. The evidence which supports this conclusion is as follows: (1) salicylates had no effect on respiration in the absence of free Ca^{2+} (i.e. in the presence of EGTA) (cf. Fig. 2), (2) inhibition of Ca^{2+} uptake by ruthenium red prevented the effects of salicylates + Ca^{2+} (see Results), as well as the possibility of Ca^{2+} cycling across the membrane, (3) Ca^{2+} alone had effects identical to those of salicylate + Ca^{2+} when incubated at high concentrations and/or long incubation times (see Results), (4) these effects depended on the presence of phosphate at concentrations (> 1 mM) similar to those required for Ca^{2+} to induce an increase in the permeability of the mitochondrial inner membrane (cf. Fig. 5 and Refs. 25–29), and (5) the reduction of intramitochondrial NAD^+, as in the presence of endogenous substrates and rotenone, prevented both the effects of salicylate + Ca^{2+}, as well as Reye's plasma and allantoin + Ca^{2+} (cf. Figs. 8 and 9), on mitochondrial function and the membrane damage induced by Ca^{2+} + phosphate [28–34].

The role of Ca^{2+} in the process of necrosis and cell death has been a subject of increasing interest in the past few years [35]. Schanne *et al.* [36] have linked the cytotoxic activity of a variety of different agents to the movement of Ca^{2+} across the plasma membrane into the cell and have concluded that an increased influx of Ca^{2+} is an essential factor in cell injury. An increased influx of Ca^{2+} into the cytoplasm would be expected to stimulate the transport of Ca^{2+} into the mitochondria and could lead to disruptions in mitochondrial structure and function as a result of the massive loading of Ca^{2+} and its storage as insoluble complexes within the matrix. Alternatively, in the presence of agents such as Reye's plasma, allantoin or salicylates, which potentiate the effects of Ca^{2+}, it could lead to an increase in the permeability of the mitochondrial inner membrane and the loss of intramitochondrial cofactors and Ca^{2+}. In either case, the energy-producing functions of the mitochondria would be disrupted, altering the energy balance of the cell as a whole.

The question then arises as to the mechanism by which salicylate and salicyl derivatives potentiate the damaging effects on the mitochondrial membrane. One possibility is that these compounds, salicylate in particular, may complex with Ca^{2+}, thus altering its partition coefficient between the membrane and the aqueous medium. Examination of the structures (cf. Fig. 10) of the salicyl compounds tested in these studies reveals that the two most toxic derivatives were those which possess a carboxyl group at the ortho position of the phenolic group (salicylate, ASA); those with a hydroxyl group in this position (salicyl alcohol, catechol) were of intermediate potency, while the amide was the least effective. Tylenol (acetaminophen), which has an acetamido group in the para position, was also only minimally effective. This suggests that the functional group may play an important role in the ability of the salicylates to bind Ca^{2+} and potentiate its effects. On the other

hand, alternative mechanisms can also be proposed. Salicylate may interact with and stimulate the action of a protein (carrier, Ca^{2+}-binding protein, etc.) directly involved in the mitochondrial uptake/release pathways for Ca^{2+}. Or, it may act simply as a permeant anion, being cotransported with Ca^{2+}.

Our results are consistent with a role for salicylates in the etiology of Reye's syndrome. It is unlikely, however, that salicylate is the only agent responsible. Indeed, allantoin, which is structurally very different than the salicylates, can also affect mitochondrial respiration in the same manner as salicylates and Reye's plasma. It seems more likely, therefore, that the toxic agent or agents which cause the mitochondrial injury in Reye's syndrome may belong to a functional class of compounds, including allantoin and salicylates, capable of enhancing the Ca^{2+}-induced damaging of the mitochondrial inner membrane. This enhancement could be produced by an appropriately high dose of any one of these agents. On the other hand, one must also consider the possibility that in the presence of two or more of these agents, each at a concentration too low to produce an effect, additive effects might occur which would push the system over the threshold of toxicity, resulting in mitochondrial damage and possible cell death.

In conclusion, we have demonstrated that preincubation of tightly-coupled rat liver mitochondria oxidizing NAD^+-linked substrates with salicylates + Ca^{2+} caused an increase in the permeability of the inner membrane and the consequent loss of intramitochondrial pyridine nucleotides. These results are virtually identical to those reported earlier [9] for the effects of Reye's plasma and allantoin + Ca^{2+} on mitochondrial metabolism. In each of these cases, the data suggest that the effect of the "toxin" (i.e. salicylate, allantoin or Reye's serum) is to potentiate the damaging effects of Ca^{2+} on mitochondrial respiration.

Acknowledgements—This work has been supported by research grants from the National Institutes of Health.

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