Studies on the Mechanism of Action of Salicylates IV: Effect of Salicylates on Oxidative Phosphorylation

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Abstract ☐ The hydrolysis of acetylsalicylic acid and the oxidative-phosphorylation uncoupling activity of salicylic acid and acetylsalicylic acid in rat liver mitochondrial preparations at pH 7.4 and 25° have been studied. The rate of hydrolysis of acetylsalicylic acid in mitochondrial preparations under these conditions is fairly slow. The uncoupling activity observed in the presence of acetylsalicylic acid is due to the salicylic acid produced during the experiment. The results obtained in the present study support some recent findings on the effect of salicylates on several other biologic activities.

Keyphrases ☐ Salicylate activity—mechanism ☐ Oxidative-phosphorylation uncoupling—salicylate effect ☐ Aspirin effect—oxidative-phosphorylation uncoupling ☐ Liver mitochondrial mixtures—aspirin hydrolysis

In a previous report the authors have shown that salicylic acid, contrary to common expectation, has higher partition-coefficient values than acetylsalicylic acid in a wide range of pH (1). In the present study it has been demonstrated that acetylsalicylic acid, unlike salicylic acid, does not uncouple oxidative phosphorylation.

Brody (2) in 1956, made the first study on the effects of salicylates on oxidative phosphorylation and found that sodium acetylsalicylate and sodium salicylate concentrations above 2×10^{-4} *M* uncoupled oxidative phosphorylation, sodium salicylate producing the greater uncoupling effect of these two compounds. The smaller extent of uncoupling in the acetylsalicylate preparations was explained by Brody as probably due to the hydrolytic amounts of salicylate produced from the hydrolysis of the acetylated compound. However, he made no actual analysis to determine the fraction of salicylic acid preexisting in his acetylsalicylic acid samples, nor did he determine the rate of hydrolysis of acetylsalicylic acid in the systems used for the study of oxidative phosphorylation. Brody's studies have been confirmed subsequently by many laboratories, most of which employed only salicylic acid in their studies (3–9). During the last decade, Smith *et al.* (10) have made extensive studies on the effects of anti-inflammatory agents on oxidative phosphorylation. Smith has implied that both salicylic and acetylsalicylic acids uncouple oxidative phosphorylation (11). Adams and Cobb also seem to share this view (12). In the present report, a definitive study was made to determine whether both salicylic acid and acetylsalicylic acid uncouple oxidative phosphorylation.

In the present study, the authors have measured the rate of hydrolysis of acetylsalicylate in rat liver mitochondrial mixtures closely approximating those of Brody and studied the relative uncoupling activities of salicylic and acetylsalicylic acids in such preparations. They then correlated the observed uncoupling effects in the acetylsalicylate mixtures with the hydrolytic amounts of salicylate produced in these mixtures.

EXPERIMENTAL

Materials and Chemicals—Livers of male, Sprague-Dawley rats weighing 200–250 g. were used in all experiments. Crystalline adenosine-5′-triphosphate (Sigma grade, disodium salt from equine muscle),[1] cytochrome c (Sigma grade, type II, from horse heart),[1] and yeast hexokinase (type III, 20 units/mg.)[1] were used. The ACS reagents,[2] anhydrous dextrose powder, potassium chloride crystals, anhydrous ether, and salicylic acid crystals, were all used. ACS spectrophotometrically pure acetylsalicylic acid[3] was also used. Magnesium chloride hexahydrate,[4] another ACS reagent, sodium pyruvate, reagent grade,[5] L-Malic acid,[6] and ACS reagents, hydrochloric acid, crystalline sodium phosphate (dibasic), and crystalline monopotassium phosphate[4] were all used.

Rate Studies on the Hydrolysis of Acetylsalicylic Acid in Rat Liver Mitochondrial Mixtures—Rats were killed by a blow to the head, drained of blood briefly, and the livers removed and placed in ice-cold, 0.25 *M* sucrose. A 10% homogenate of the liver was made in 0.25 *M* sucrose using the Dounce homogenizer (13). The mitochondrial fraction was then isolated according to the procedure of Schneider and Hogeboom (14), using 0.25 *M* sucrose. The mitochondrial fraction was suspended in cold 1.7×10^{-2} *M* phosphate buffer, pH 7.4 (2 ml. buffer/4 g. fresh liver tissue) and 2 ml. of the resulting suspension was immediately added to a cold Pyrex melting-point flask containing other components of Brody's mitochondrial mixture (2). The flask with contents was placed into a constant-temperature (25°) water bath, equipped with a shaking mechanism, and allowed to equilibrate with agitation for 5 min. Individual solutions of yeast hexokinase-glucose and of freshly prepared acetylsalicylic acid in 1.7×10^{-2} *M* phosphate buffer, pH 7.4, both of which had equilibrated for 5 min. in the 25° bath, were added to the flask in the 25° bath and a 1-ml. sample (0-time sample) was immediately removed. The shaker was engaged and 1-ml. samples were removed at 10-min. intervals over a period of 70 min.

The final concentrations of the components for the suspensions under study were: KCl 1.7×10^{-2} *M;* $MgCl_2$, 1.3×10^{-2} *M;* ATP, 2.8×10^{-3} *M;* sodium pyruvate, 1.5×10^{-2} *M;* malate, 2.0×10^{-3} *M;* cytochrome c, 1.0×10^{-5} *M;* yeast hexokinase, 16.7 units/ml.; glucose, 3.3×10^{-2} *M;* NaF, 1.5×10^{-2} *M;* mitochondrial preparation, 0.22 ml./ml.; acetylsalicylate, 1×10^{-4} *M* to 20×10^{-4} *M*.

The salicylic acid was extracted from the 1-ml. samples, removed at various times, using a modification of the method of Chirigos and Udenfriend (15). The 1-ml. fraction was immediately placed into a 50-ml. ground-glass-stoppered centrifuge tube, containing 1 ml. of 2 *N* HCl and 25 ml. of anhydrous ether, which had equilibrated in an ice bath. The tube was vigorously shaken 100 times and replaced in the ice bath. After the layers separated, 10 ml. of the ether layer was removed and added to 10 ml. of 0.1 *M* phosphate buffer, pH 7.4, in a ground-glass-stoppered, 50-ml. centrifuge tube

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Baker analyzed, J. T. Baker Chemical Co., Phillipsburg, N. J.
[3] City Chemical Co., New York, N. Y.
[4] Allied Chemical, General Chemical Division, New York, N. Y.
[5] Nutritional Biochemical Corp., Cleveland, Ohio.
[6] Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.

Table I—Percent Hydrolysis of Aspirin at Various Concentrations in Rat Liver Mitochondrial Reaction Mixtures at 25°, pH 7.4

Time, min.	Initial ASA Concn.			
	1×10^{-4} *M*	5×10^{-4} *M*	10×10^{-4} *M*	20×10^{-4} *M*
0[a]	3.50 ± 0.35	4.32 ± 0.56	4.02 ± 0.83	4.30 ± 0.72
10	1.86 ± 0.94	1.33 ± 0.38	1.21 ± 0.40	1.88 ± 0.65
20	3.21 ± 0.58	3.38 ± 0.19	2.89 ± 0.28	3.67 ± 0.84
30	4.59 ± 0.60	4.58 ± 0.76	4.24 ± 0.91	5.34 ± 1.33
40	6.28 ± 1.03	5.69 ± 0.86	5.66 ± 0.83	6.86 ± 1.70
50	7.98 ± 1.51	7.52 ± 1.53	6.83 ± 0.76	8.26 ± 2.27
60	9.94 ± 2.16	9.18 ± 0.43	7.82 ± 0.99	9.57 ± 2.67
70	10.92 ± 1.99	11.05 ± 0.47	8.82 ± 1.21	10.68 ± 2.81

[a] Salicylic acid content in the reaction mixture at zero time serves as the blank.

and the mixture shaken vigorously 100 times. When the layers separated, appropriate aliquots of the buffer layer were placed into each of two 25-ml. volumetric flasks. To one flask was added 0.1 ml. of concentrated NH_4OH and the mixture heated on the steam bath for 5 min., to hydrolyze all remaining acetylsalicylate, and cooled. Dry nitrogen was bubbled through the second flask for 2 min., to remove residual ether, and both flasks were filled to volume with 0.1 *M* phosphate buffer, pH 7.4. Both solutions were analyzed for salicylic acid in the spectrophotofluorometer.

Uncoupling Studies—Rat liver mitochondria was isolated and the mitochondrial suspensions were prepared according to the procedure described above. The mitochondrial mixtures in which these studies were made and the procedures used were essentially those of Brody with minor modifications. To the main compartment of double-armed Warburg vessels were added the cold reaction mixture and cold, freshly prepared salicylic acid and acetylsalicylic acid at various concentrations, or 1.7×10^{-2} *M* phosphate buffer, pH 7.4. In one sidearm was added 0.4 ml. of 5.5 *N* HCl, and to the other sidearm was added 0.2 ml. of yeast hexokinase in glucose solution in 1.7×10^{-2} *M* phosphate buffer, pH 7.4. A small roll of filter paper was placed in the center cell containing 0.2 ml. of 2 *N* KOH. Additions of acetylsalicylic and salicylic acids, made up in 1.7×10^{-2} *M* phosphate buffer, pH 7.4, were timed such that one would obtain minimum hydrolysis of aspirin prior to the experimental measurements. The mitochondrial suspension was the last component added to the main compartment. The flasks were attached to manometers and placed into the 25° constant-temperature bath and the shaker engaged. Stopcocks were opened to the atmosphere. After a 10-min. period of equilibration, both the hexokinase-glucose and the 5.5 *N* HCl solutions were tipped into the flasks used to determine initial phosphate concentrations. Hexokinase-glucose solution was tipped into the other flasks and the stopcocks closed to the atmosphere. The reaction mixtures contained the same components, at the same concentrations, with the exception of aspirin and salicylic acid, as the reaction mixture described for the hydrolysis studies above. The volume of the mixture in the main compartment during the reaction was 1.8 ml. The total volume of solution in the Warburg vessels during the uncoupling studies was 2.4 ml. Oxygen uptake was measured for a period of 30 min. at 25° and the reaction was stopped by tipping in the 5.5 *N* HCl from the sidearm. Immediately after adding the 5.5 *N* HCl to vessels containing acetylsalicylate, the vessel was quickly removed from the manometer, filter paper removed, center well sponged out with swabs and 5 ml. of 1 *N* HCl added. The resultant was swirled gently and a suitable aliquot was introduced into 25 ml. of cold anhydrous ether in a 50-ml. ground-glass-stoppered centrifuge tube and the procedure described above for the determination of salicylic acid was followed. The remainder of the acidic acetylsalicylate reaction mixture was centrifuged and a suitable aliquot of the clear supernatant was analyzed for inorganic phosphate according to the procedure of Fiske and Subbarow (16). After tipping the 5.5 *N* HCl into the control flasks and flasks containing salicylic acid, they were removed from the manometer, the filter paper removed from the center well, the center well swabbed, and 5 ml. of 1 *N* HCl was added. The mixtures were centrifuged and the clear supernatant was analyzed for phosphate as above.

RESULTS AND DISCUSSION

Rate Studies on the Hydrolysis of Acetylsalicylic Acid—The results of the rate studies for the hydrolysis of acetylsalicylic acid are summarized in Table I, in which the authors have tabulated the percent hydrolysis at various initial concentrations. A plot of the log of percent of hydrolysis *versus* time yields essentially a straight line in every one of the four concentrations of acetylsalicylic acid studied. Figure 1 is a representative plot. The linearity of the plot shows that the reaction for the hydrolysis of acetylsalicylate in rat liver mitochondrial mixture is approximately first order. Edwards (17) has shown that the nonenzymatic hydrolysis of acetylsalicylate in buffer systems at this pH is approximately first order. The mean first-order velocity constant obtained from consideration of all the initial concentrations of acetylsalicylate was found to be 1.6×10^{-3} min.$^{-1}$, whereas the first-order velocity constant of Edwards for the hydrolysis of acetylsalicyate in aqueous solutions was calculated to be 0.192×10^{-3} min.$^{-1}$ at pH 7.4 and 25°. The fact that the first-order velocity constant obtained here is approximately eight times larger indicates that there is some limited enzymatic hydrolysis to these preparations.

Uncoupling Studies—The uncoupling effects of acetylsalicylate and salicylic acid at various concentrations in rat liver mitochondrial mixtures at 25° are shown in Table II. No uncoupling could be seen with acetylsalicylate concentrations of 7.5×10^{-4} *M* and below. Concentrations of salicylic acid above 20×10^{-4} *M* completely abolished phosphate uptake.

A plot of the P/O (the ratio of gram-atoms of inorganic phosphate uptake and gram-atoms of oxygen utilized which is a measurement of oxidation phosphorylation) *versus* concentrations of acetylsalicylic and salicylic acids using the authors' data and the data of Brody are shown in Fig. 2. It is clear that the comparative plot of the data of Brody and that from the present studies for the uncoupling effect of acetylsalicylic acid does not show close agreement.

If, using the data obtained in the present study, one corrects for the uncoupling effect due to the hydrolytic amounts of salicylic acid present in the mixtures containing acetylsalicylic acid, one finds that acetylsalicylic acid does not uncouple. This is shown in Fig. 3.

If, using Brody's plot of P/O *versus* concentration of acetylsalicylic and salicylic acids, one draws lines parallel to the concentration axis such that they bisect both the salicylic and acetylsalicylic acid curves, one is able to obtain, graphically the approximate concentration of salicylic acid necessary to produce the uncoupling effect

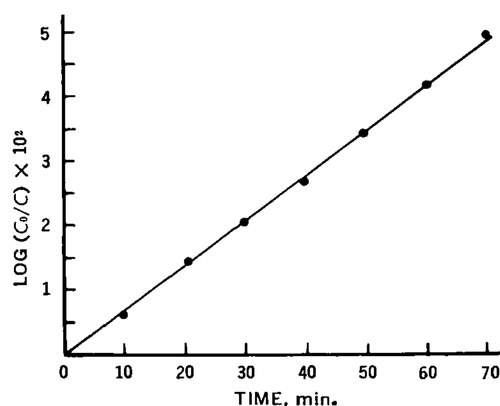

Figure 1—*Hydrolysis of ASA* (1×10^{-4} *rat liver mitochondrial mixtures at pH 7.4, 25°.* C_0 = *initial ASA concentration,* C = *ASA concentration at time,* t.

Table II—*Effects of Acetylsalicylic (ASA) and Salicylic (SA) Acids on Oxidative Phosphorylation in Rat Liver Mitochondria at 25°, pH 7.4*

Drug	Concn., *M*	P/O	Control, %	Hydrolysis, %	SA Concn. $\times 10^{-4}$ *M*
None	—	2.59 ± 0.10	100	—	—
ASA	30×10^{-4}	1.86 ± 0.40	70.94 ± 14.92	10.73 ± 2.38	3.22 ± 0.71
	20×10^{-4}	2.22 ± 0.14	85.07 ± 4.66	11.03 ± 0.89	2.21 ± 0.18
	10×10^{-4}	2.44 ± 0.14	92.61 ± 5.51	10.44 ± 1.35	1.04 ± 0.14
SA	10×10^{-4}	0.96 ± 0.35	37.13 ± 11.51	—	—
	7.5×10^{-4}	1.20 ± 0.50	46.35 ± 16.60	—	—
	5×10^{-4}	1.43 ± 0.09	54.80 ± 3.59	—	—
	2.5×10^{-4}	1.85 ± 0.16	71.80 ± 6.73	—	—

seen in Brody's preparations of acetylsalicylic acid. Using this procedure one finds that approximately 70% of the acetylsalicylate must be hydrolyzed to produce the uncoupling effect observed in his preparations. Since the results of this study do not show such a rapid rate of hydrolysis in similar preparations, there must be other possibilities to consider. At least three possibilities are apparent: (*a*) Brody's original samples of acetylsalicylic acid contained large quantities of salicylic acid; (*b*) a fairly large amount of salicylic acid was produced in the preparation of the sodium salt; (*c*) Brody's preparations contained fairly large quantities of the esterases which hydrolyze acetylsalicylate and a large amount of salicylic acid was produced in the incubation. Since Brody made no analysis in his preparations, one cannot determine which of these possibilities carries the greatest weight.

The uncoupling results obtained in the present study support the findings obtained by other workers who studied the effect of salicylates on various biological activities. Acetylsalicylate does not decrease the incorporation of glucose-^{14}C, acetate-^{14}C, and $^{35}SO_4^=$ into mucopolysaccharide sulfates in cartilage and cornea *in vitro*, effects normally produced by salicylate and other uncoupling agents (18). Recently, Levy *et al.* (19) have shown that salicylate (15 m*M*) significantly inhibits the active transport of L-tryptophan across the small intestine of the hamster; aspirin (15 m*M*) has no inhibitory effect. They also found that salicylate (15 m*M*) prevents the active transport of the amino acid, L-tryptophan, across the small intestine of the rat, while aspirin (15 m*M*) appears to inhibit somewhat, but does not prevent, this process. Active transport is an energy-requiring process. Inhibition of the process is to imply uncoupling of oxidative phosphorylation.

However, even though there is evidence to indicate that the salicylates might exert their anti-inflammatory effects by uncoupling oxidative phosphorylation, and that depression of this energy-yielding process might be a general mechanism by which a large number of anti inflammatory and antirheumatic drugs exert their effects, one has to contend with the fact that 2,4-dinitrophenol has not been shown to have either anti-inflammatory or antirheumatic effects, even though it is a much more potent uncoupling agent than salicylic acid. In addition, acetylsalicylate is generally considered to be a more potent anti-inflammatory agent than salicylate (20), yet the results here indicate that acetylsalicylate does not uncouple oxidative phosphorylation.

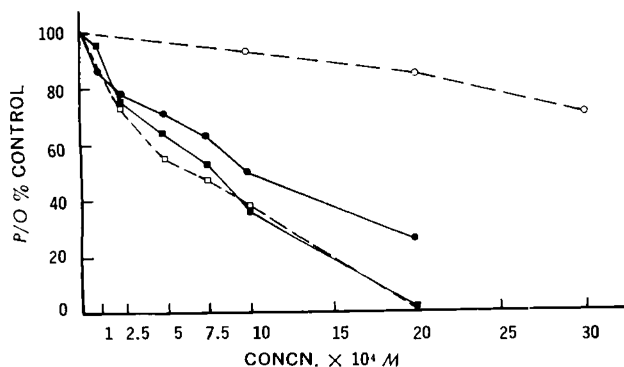

Figure 2—*Effects of ASA on SA on oxidative phosphorylation in rat liver mitochondrial mixtures at pH 7.4, 25°. Comparative plot of the data of Brody and the results of the present studies. Key:* ●, *Brody's data for ASA (2);* ○, *results of the present studies for ASA;* ■, *Brody's data for SA (2);* □, *results of the resent studies for SA.*

Perhaps it may be proposed that the importance of the uncoupling activity of the salicylates to their pharmacological activities can better be determined through a consideration of the structural requirements for uncoupling activity and a determination of through what mechanism and at what site they exert this effect in mitochondria. Some workers have explored these areas. Panagopoulos (6) and Whitehouse (18) are of the opinion that the presence of a free phenolic hydroxyl group in the salicylate moiety is necessary for uncoupling activity. The results obtained here seem to support this contention. However, Bosund (8) has shown that benzoic acid, in high concentrations, inhibits oxidative phosphorylation in rat liver mitochondria. In addition, Bosund had reported that 4-hydroxysalicylic, 5-hydroxysalicylic, and 4-aminosalicylic acids do not uncouple oxidative phosphorylation.

If the hydroxyl of the carboxy group in salicylic acid is replaced with methyl, uncoupling activity is abolished (21). However, it has been reported that methylbenzoate inhibits oxidative phosphorylation to about the same extent as salicylic acid in rat liver mitochrondria (8).

It is interesting to note that 2-mercaptobenzoic (thiosalicylic) acid is a more potent uncoupler than salicylic acid (9, 22). Burke and Whitehouse (21) explain this greater potency on the basis of the thio compound's greater lipophilic character and its large pKa (about 4). They compare the pKa's of the potent enolic uncoupling agents with lipophilic character, phenylbutazone (pKa = 4.5), 2,4-dinitrophenol (pKa = 4.0), and 2-phenyl-indan-1,3-dione (pKa = 4.2). Since it is the carboxyl group of 2-mercaptobenzoate which has a pK of about 4, these workers suggest that the *ortho* thio group may not be involved in the uncoupling, although it may confer lipid solubility through intramolecular hydrogen bonding.

There is seemingly no clear correlation, based on present data, between various salicylate derivatives and uncoupling activity.

The locus of the uncoupling action of the salicylates is equally unclear. Salicylates stimulate mitochondrial adenosine triphosphatase (23). Charnock and Opit (24), on the basis of the effects of

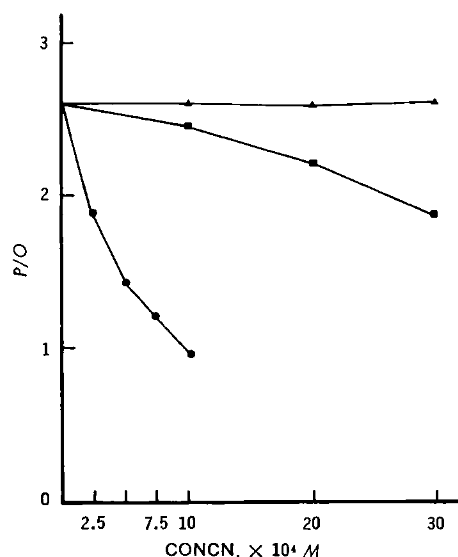

Figure 3—*The effects of ASA and SA on oxidative phosphorylation in rat liver mitochondrial mixtures at pH 7.4, 25°. Key:* ●, *salicylic acid;* ■, *observed curve for ASA;* ▲, *corrected curve for ASA hydrolysis.*

salicylate on adenosine triphosphatase activity at different pH values in fresh and aged mitochondria, suggest that these compounds uncouple by acting at the mitochondrial membrane, increasing the membrane's permeability to ATP, thus stimulating adenosine triphosphatase activity and increasing the breakdown of ATP.

Smith (22) has suggested that the formation of a salicoyl phosphate followed by an intramolecular rearrangement to salicyl phosphate followed by hydrolysis could be a mechanism by which salicylates might act as phosphate acceptors and hence uncouple.

These workers ignore the electron transport chain itself. However, some workers have presented evidence that the phosphorylations associated with the entire respiratory chain may be affected by salicylates (4, 7). Another has reported evidence that the terminal phosphorylation step may be more sensitive to the uncoupling effect of the salicylates (25). However, until the process of oxidative phosphorylation is clearly elucidated, the locus of the uncoupling action of any compound must necessarily remain speculative.

In conclusion, these studies have shown, conclusively, that acetylsalicylic acid, as the intact molecule, does not uncouple oxidative phosphorylation. They, therefore, demonstrate still another biological activity where acetylsalicylic acid and salicylic acid differ.

SUMMARY

The hydrolysis of acetylsalicylic acid and the oxidative phosphorylation uncoupling activity of acetylsalicylic and salicylic acids have been studied in rat liver mitochondrial preparations at pH 7.4 and 25°. The rate of hydrolysis of acetylsalicylic acid under these conditions is fairly slow, approaching the rate of hydrolysis in nonbiological systems at room temperature.

It was shown that the uncoupling in the acetylsalicylate preparations was due to the salicylic acid produced during the experiment. The uncoupling results compare favorably with results obtained by other workers using other biological systems.

Theories on the consequences, structural requirements, and locus of salicylate uncoupling are discussed.

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