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Effect of Salicylate on Oxidative Phosphorylation and Respiration of Mitochondrial Fragments

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1. The effects of salicylate on oxidative phosphorylation and respiration were investigated in liver and brain mitochondria, and in sonically prepared mitochondrial fragments. 2. Salicylate was shown to uncouple oxidative phosphorylation in mitochondrial fragments, as well as in intact mitochondria. The effects of salicylate on mitochondria and oxidative phosphorylation resemble those produced by dinitrophenol. 3. The quantitative effects of salicylate on respiration *in vitro* were shown to be complex. The final effect may be the resultant of an interaction of a multiplicity of factors, such as the tissue, the substrate, mitochondrial and extramitochondrial influences, and the direct effect on the respiratory chain itself.

Brody (1956) reported that salicylate uncouples oxidative phosphorylation; subsequently many investigators have studied the uncoupling effect of the drug. This influence on energy metabolism is employed to explain some of the clinical effects and untoward manifestations associated with salicylate therapy (Brody, 1956; Whitehouse, 1964). The exact mechanism of the uncoupling, as well as the primary subcellular site of action, however, has yet to be defined unequivocally. For example, Falcone (1959) reported that salicylate stimulated mitochondrial adenosine-triphosphatase activity, and Charnock & Opit (1962) observed that salicylate facilitates the enzymic breakdown of ATP by affecting the permeability of the mitochondrial membrane. From these observations, it is conceivable that the salicylate-induced uncoupling is the consequence of an elevated adenosine-triphosphatase activity and is mediated by alterations in the structure of the mitochondrion (Charnock & Opit, 1962).

Other evidence, however, indicates that salicylate may uncouple by a direct action on the oxidative-phosphorylation reactions. Jeffrey & Smith (1959) found that salicylate, like 2,4-dinitrophenol, retards mitochondrial swelling. Similarly, Penniall (1958) demonstrated that salicylate, again like dinitrophenol, uncouples at each phosphorylation site. When such observations are considered in the light of current concepts of the direct action of dinitrophenol on phosphorylation, there is some support for the interpretation that salicylate also uncouples by a direct action on the phosphorylation process.

The approach in the present study was to compare the effect of salicylate on intact mitochondria with the effect on a simpler model, namely, mitochondrial fragments prepared by sonic disruption. These fragment preparations, resistant to the uncoupling action of Ca^{2+} (McMurray, Maley & Lardy, 1958) and 1 mM-thyroxine (J. T. Miyahara & R. Karler, unpublished work), provided an opportunity to determine whether the uncoupling action of salicylate requires the presence of an intact mitochondrial membrane or whether the action is of a direct nature on the enzyme system present in the mitochondrial fragments. In addition to the phosphorylation experiments, the effect of salicylate on the oxidation rate of various substrates was compared in both intact mitochondria and mitochondrial fragments from liver and brain.

METHODS

Liver and brain mitochondria were isolated from adult male Sprague–Dawley rats by differential centrifugation (Karler, Sulkowski & Miyahara, 1965). Mitochondrial fragments were prepared sonically in a 9 kcyc./sec. Raytheon oscillator as reported by McMurray *et al.* (1958) with two modifications: mitochondria were subjected to sonic vibrations for a 20 sec. duration and the centrifugation time of the crude extract at 105000*g* was decreased to 30 min. The two variations produced maximal P/O ratios for liver fragments and adequately measurable ratios for brain fragments.

Oxygen consumption was measured by standard manometric techniques. For intact mitochondria, the reaction medium consisted of 40 μmoles of potassium phosphate buffer, pH 7·4, substrate (40 μmoles of sodium pyruvate, 20 μmoles of sodium succinate, or 40 μmoles of sodium β-hydroxybutyrate + 3 μmoles of NAD^+), 5 μmoles of ATP, 5 μmoles of $MgCl_2$, 50 μmoles of glucose, 0·8 mg. of hexokinase, 150 μmoles of tris–HCl buffer, pH 7·4, 250 μmoles of sucrose, 1·0 ml. of mitochondrial suspension (1 ml. of suspension in 0·25 M-sucrose was equivalent to 250 mg. wet wt.

of tissue mash), and sodium salicylate in 0·1 M-tris–HCl buffer, pH 7·4. For fragment studies, each flask contained 15 μmoles of potassium phosphate buffer, pH 7·4, substrate (20 μmoles of sodium succinate, 40 μmoles of sodium β-hydroxybutyrate + 3 μmoles of NAD^+, or 5 μmoles of NADH), 10 μmoles of ATP, 15 μmoles of $MgCl_2$, 50 μmoles of glucose, 0·8 mg. of hexokinase, 150 μmoles of tris–HCl buffer, pH 7·4, 10 μmoles of KF, 1·5 ml. of fragment suspension, and 0·1 ml. of salicylate. The medium for fragments differed from that for mitochondria because the higher concentrations of $MgCl_2$ and ATP and the inclusion of KF were required for optimum P/O ratios in the fragments. The final volume for all P/O determinations was 3·3 ml. and the temperature 30°; air was used as the gas phase.

The flasks were equilibrated for 8 min., and P/O determinations were made in the following 15 min., after which 0·7 ml. of 50% (w/v) trichloroacetic acid was added. When succinate was the substrate with intact liver mitochondria a 10 min. period was used for determination of the P/O ratio. Inorganic phosphate was determined colorimetrically by means of the molybdate–*p*-semidine reaction, a modification of the method of Dryer, Tammes & Routh (1957), described by Knights, MacDonald & Ploompuu (1962).

Sodium salicylate (analytical grade; Mallinckrodt Chemical Works, New York, N.Y., U.S.A.) was used in all drug studies. The drug was added to the reaction medium in a 0·1 M-tris–HCl buffer, pH 7·4. For the P/O studies, hexokinase (type III), ATP (disodium salt), NAD^+ and NADH were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.), and *p*-semidine (*N*-phenyl-*p*-phenylenediamine monohydrochloride) from Distillation Products Industries (Rochester, N.Y., U.S.A.). All other reagents were of analytical grade.

RESULTS

Effect of salicylate on oxidative phosphorylation and respiration of liver mitochondria. Table 1 shows salicylate effects on liver mitochondria oxidizing pyruvate, succinate and β-hydroxybutyrate respectively. As reported by others and shown in Table 1, there is a direct relation between drug concentration and effect on the uptake of inorganic phosphate and on the P/O ratio for all three substrates studied. A low concentration of salicylate (0·01 mM) slightly affected phosphorylation and the P/O ratio, whereas a high concentration (1 mM) markedly decreased both values. Oxidative phosphorylation was completely uncoupled by 10 mM-salicylate irrespective of substrate. The additional phosphate liberated (shown as negative uptake in Table 1) at these high concentrations of salicylate probably resulted from the breakdown of the added ATP. The oxidation of pyruvate and succinate by liver mitochondria was significantly depressed by 1 mM-salicylate, whereas oxygen consumption of liver mitochondria with β-hydroxybutyrate as substrate was not affected to the same degree at this concentration.

Effect of salicylate on oxidative phosphorylation and respiration in brain mitochondria. Results of experiments conducted on brain mitochondria are illustrated in Table 2. As with liver, phosphorylation and P/O ratios were similarly affected by salicylate in that both were decreased by increasing salicylate concentrations. Complete uncoupling and increase of inorganic phosphate concentration were also observed at 10 mM concentration. The effect on respiration of brain mitochondria, however, differed from that seen with liver preparations. As reported by Brody (1956), a salicylate concentration (10 mM) that completely dissociated oxidation from phosphorylation stimulated oxygen utilization in brain when pyruvate was the sub-

Table 1. *Effect of salicylate on oxidative phosphorylation by liver mitochondria*

Experimental details are given in the text.

Substrate	Concn. of salicylate (mM)	No. of experiments	P_i uptake (μmoles/15 min.)	O_2 uptake (μg.atoms of O/15 min.)	P/O ratio ± S.E.M.
Pyruvate	0 (control	7	29·56	12·01	2·46 ± 0·06
	0·01	6	26·16	11·79	2·22 ± 0·04
	0·1	5	24·07	11·52	2·09 ± 0·05
	1·0	5	5·94	7·84	0·76 ± 0·05
	10	5	−7·87	3·04	0
Succinate	0 (control)	5	17·09*	9·19*	1·86 ± 0·04
	0·01	5	16·90*	9·56*	1·77 ± 0·04
	0·1	5	12·26*	8·30*	1·47 ± 0·06
	1·0	5	2·19*	6·01*	0·36 ± 0·05
	10	5	−3·71*	3·18*	0
β-Hydroxybutyrate	0 (control)	6	15·98	8·09	1·98 ± 0·07
	0·01	5	13·41	8·13	1·65 ± 0·03
	0·1	5	10·45	7·74	1·35 ± 0·08
	1·0	5	3·54	7·01	0·50 ± 0·10
	10	6	−4·26	4·63	0

* Uptake values for a 10 min. period.

Table 2. *Effect of salicylate on oxidative phosphorylation by brain mitochondria*

Experimental details are given in the text.

Substrate	Concn. of salicylate (mM)	No. of experiments	P_i uptake (μmoles/15 min.)	O_2 uptake (μg.atoms of O/15 min.)	P/O ratio ± S.E.M.
Pyruvate	0 (control	5	8·93	4·55	1·96 ± 0·09
	0·01	5	8·19	4·39	1·87 ± 0·09
	0·1	5	6·72	4·78	1·41 ± 0·07
	1·0	5	2·92	5·11	0·57 ± 0·06
	10	5	−2·62	5·79	0
Succinate	0 (control)	5	11·39	6·08	1·87 ± 0·04
	0·01	4	9·63	5·60	1·72 ± 0·03
	0·1	4	6·86	4·94	1·38 ± 0·06
	1·0	5	2·93	4·13	0·71 ± 0·03
	10	3	−3·98	2·83	0
β-Hydroxybutyrate	0 (control)	5	6·23	2·98	2·09 ± 0·09
	0·01	5	6·08	3·26	1·87 ± 0·09
	0·1	5	5·36	3·19	1·68 ± 0·27
	1·0	5	2·60	2·99	0·87 ± 0·06
	10	5	−3·36	2·33	0

Table 3. *Effect of salicylate on oxidative phosphorylation by mitochondrial fragments*

Experimental details are given in the text.

Source of mitochondrial fragments	Substrate	Concn. of salicylate (mM)	No. of experiments	P_i uptake (μmoles/15 min.)	O_2 uptake ± S.E.M. (μg.atoms of O/15 min.)	P/O ratio ± S.E.M.
Liver	β-Hydroxybutyrate	0 (control)	8	2·74	3·49	0·77 ± 0·06
		0·01	5	2·21	4·48	0·49 ± 0·05
		0·1	5	0·49	3·66	0·13 ± 0·11
		1·0	5	−1·09	2·90	0
		10	5	−1·63	2·05	0
Liver	Succinate	0 (control)	5	3·84	6·23	0·62 ± 0·03
		0·01	4	2·78	6·24	0·44 ± 0·04
		0·1	4	1·58	5·51	0·29 ± 0·05
		1·0	3	−0·97	5·63	0
		10	3	−1·72	2·96	0
Brain	NADH	0 (control)	5	3·28	3·77 ± 0·23	0·87
		0·01	5	1·96	3·73 ± 0·30	0·53
		0·1	5	1·24	3·34 ± 0·08	0·37
		1·0	5	−0·30	3·29 ± 0·17	0
		10	5	−1·38	2·23 ± 0·42	0
Liver	NADH	0 (control)	5	3·36	4·92 ± 0·26	0·69
		0·01	5	2·77	5·01 ± 0·29	0·55
		0·1	5	1·45	4·23 ± 0·26	0·34
		1·0	5	−0·44	4·43 ± 0·20	0
		10	5	−2·22	4·72 ± 0·59	0

strate. With succinate and β-hydroxybutyrate, brain mitochondria reacted to salicylate as did liver mitochondria, by exhibiting depression of oxygen uptake at comparable concentrations.

Effect of salicylate on oxidative phosphorylation of liver mitochondrial fragments. Experiments on liver mitochondrial fragments designed to establish whether the intact membrane of the mitochondrion is necessary for salicylate action are presented in Table 3. The results obtained with succinate and β-hydroxybutyrate are presented; the sonically prepared fragments did not oxidize pyruvate. As

illustrated in Table 3, both the uptake of inorganic phosphate and the P/O ratio were markedly depressed by 0·1 mM-salicylate. Moreover, complete uncoupling of oxidative phosphorylation was observed at a concentration of 1 mM, compared with 10 mM for a similar effect with non-disrupted mitochondria. The effect of salicylate on liver mitochondrial respiration is included in Table 3. With β-hydroxybutyrate as substrate, a low concentration (0·01 mM) of salicylate enhanced oxygen consumption, whereas with succinate as substrate the same concentration had practically no effect on the rate of oxygen utilization. At higher concentrations, oxidation of both substrates was appreciably diminished.

Effect of salicylate on the respiration of mitochondrial fragments. From the above studies it is not possible to determine the direct effect of salicylate on the reactions of the electron-transport chain. To obtain this information, experiments were undertaken to measure the effect of salicylate on brain and liver mitochondrial fragments with NADH as substrate. The use of NADH as substrate was intended to circumvent any indirect effects of salicylate on respiration that may be mediated by actions on systems outside the electron-transport chain. Therefore, with the use of mitochondrial fragments and NADH, the direct effect of salicylate on the respiratory chain could be observed (Table 3). The P/O ratios and phosphate-uptake values are included to demonstrate that they were also affected in these systems. The respiration of brain fragments was decreased from 3·77 to 2·23 μg.atoms by 10 mM-salicylate. Lower concentrations, however, had little influence on oxygen uptake by these fragments. With liver fragments, in contrast with brain fragments, salicylate had no appreciable effect on the oxidation rate of NADH, even at high, uncoupling, concentrations.

DISCUSSION

The results of the present studies on the effects of salicylate on oxidative phosphorylation in intact liver and brain mitochondria are in accord with those reported by Brody (1956), Penniall, Kalnitsky & Routh (1956) and Jeffrey & Smith (1959). In both tissues, regardless of the substrate, increasing concentrations of salicylate produced a progressive decrease in phosphate uptake and a concomitant decrease in the P/O ratio. In all instances, oxidative phosphorylation was completely uncoupled at a concentration of 10 mM. At this concentration the adenosine-triphosphatase activity became apparent, as is indicated by an increase in the total inorganic phosphate in the system. The increase in inorganic phosphate was probably a consequence of the hydrolysis of exogenous ATP.

The results obtained from fragment studies on oxidative phosphorylation also indicate that salicylate, in contrast with Ca^{2+} and thyroxine, uncouples this type of sonically treated preparation. Again, increasing concentrations of salicylate produce a progressive decrease in phosphate uptake and in P/O ratio. It appears that the fragments are more sensitive to the uncoupling action of salicylate because they are completely uncoupled by a concentration of 1 mM, rather than 10 mM required for a similar effect in intact mitochondria. The difference in sensitivity to salicylate may be more apparent than real because several factors may be involved. First, if the intact mitochondrial membrane acts as a barrier through which salicylate must pass before any action occurs, disruption of the membrane would make the receptor sites more accessible, thereby accounting for the increase in sensitivity in the fragments. Secondly, the mitochondrial and fragment preparations are not necessarily equivalent in terms of total number of reaction sites. The yield of fragments from sonically treated mitochondria is not known. Further, it has been reported that the sonically prepared fragments possess only the first two phosphorylation sites (McMurray *et al.* 1958; Kielley & Bronk, 1958; Gregg, 1963). In addition, of the two phosphorylating sites present in the fragments, site 2 apparently contributes the greater portion of the phosphate incorporation (McMurray *et al.* 1958; Kielley & Bronk, 1958; Gregg, 1963). This fact, combined with the report by Penniall (1958) that site 2 is the most vulnerable to the uncoupling action of salicylate, provides another possible explanation for the increase in sensitivity of the mitochondrial fragments to the uncoupling action of salicylate.

The ability of salicylate to uncouple mitochondrial fragments indicates that the uncoupling effect is not necessarily dependent on the intact mitochondrial membrane. In this respect salicylate action in these preparations is similar to that of dinitrophenol, but may differ from that of Ca^{2+} and thyroxine. These uncoupling agents do not uncouple oxidative phosphorylation in all fragment preparations reported. Different preparative procedures apparently result in different responses to Ca^{2+} and thyroxine (Cooper & Lehninger, 1956; Kielley & Bronk, 1957; Park, Meriwether & Park, 1958; McMurray *et al.* 1958; Penefsky, Pullman, Datta & Racker, 1960; Gregg, 1963). Variability in the uncoupling effect of dinitrophenol, however, has not been reported. The preparation described in the present work is not uncoupled by thyroxine, but is uncoupled by salicylate. Therefore, on the basis of common effects on swelling, on ability to affect the entire respiratory chain, and on uncoupling mitochondrial fragments, salicylate resembles

Table 4. *Summary of the effects of salicylate on respiration*

Effects of salicylate were measured in the concentration range 0·01–50 mM for homogenates, and in the concentration range 0·01–10 mM for mitochondria and mitochondrial fragments.

Tissue preparation / Substrate ...	... Pyruvate	Succinate	β-Hydroxybutyrate	NADH
Liver				
Homogenate	Stimulation followed by inhibition	–	–	–
Mitochondria	Inhibition	Inhibition	Inhibition	–
Fragments	–	Inhibition	Stimulation followed by inhibition	No effect
Brain				
Homogenate	Stimulation followed by inhibition	–	–	–
Mitochondria	Stimulation	Inhibition	Stimulation followed by inhibition	–
Fragments	–	–	–	Inhibition

dinitrophenol, which is believed to act directly on the phosphorylation process.

The effects of salicylate on respiration, summarized in Table 4, appear to be extremely complex. A toxic dose of salicylate *in vivo* characteristically produces stimulation followed by depression of respiration. A similar effect is seen with increasing salicylate concentrations in both liver and brain homogenates metabolizing certain substrates; a concentration of 10 mM, which completely uncouples oxidative phosphorylation in mitochondria, markedly stimulates the respiratory rate of liver and brain homogenates respiring on pyruvate (T. S. Sulkowski & R. Karler, unpublished work). Under identical conditions, the respiratory rate of isolated brain mitochondria also increases, but that of liver mitochondria decreases greatly. Therefore it appears that, at least with liver, extramitochondrial factors may be involved in determining the effect of salicylate on respiration. In addition, the substrate also appears to influence the effect on respiration (Nitzescu & Cosma, 1923). Brain mitochondrial respiration is stimulated when pyruvate is the substrate, but it is depressed when succinate or β-hydroxybutyrate is used.

From the studies of the effect of salicylate on the respiratory rate of tissue homogenates and isolated mitochondria, it is difficult to determine the direct effect on the respiratory chain. The use of mitochondrial fragments oxidizing NADH, however, provides an opportunity to determine the direct effect of salicylate on the electron-transport complex. These studies indicate that the effects observed do not necessarily coincide with those observed in intact mitochondria or in fragments metabolizing substrates other than NADH. For example, salicylate does little to the respiratory rate of liver fragments oxidizing NADH; however, respiration in the presence of succinate is markedly depressed and in the presence of β-hydroxybutyrate is stimulated at low doses and depressed at high concentrations. In contrast with liver fragments, brain fragments respiring on NADH are depressed. Therefore the direct effect of salicylate on the electron-transport reactions is different in the two tissues studied.

In general the effect of salicylate on respiration *in vitro* is obviously complex. The final effect may be the resultant of an interaction of a multiplicity of factors, such as the tissue, the substrate, mitochondrial and extramitochondrial influences, and the direct effect on the respiratory chain itself.

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