Some Effects of Salicylate on Mitochondria from Rat Liver

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Salicylate causes an increased oxygen consumption, a rapid disappearance of glycogen and a reduction of the contents of creatine phosphate and adenosine triphosphate in the isolated rat diaphragm, and it was suggested that these effects were due to interference with oxidative-phosphorylation reactions in the tissue (Smith & Jeffrey, 1956). This paper is concerned with the action of salicylate on oxidative-phosphorylation processes in mitochondria prepared from rat liver. The results confirm the observations of Brody (1956), Penniall, Kalnitsky & Routh (1956) and Penniall (1958) that salicylate uncouples the phosphorylations associated with the oxidation of various substrates in respiring mitochondrial preparations from rat liver, kidney and brain. With succinate or β -hydroxybutyrate as substrate, 1–5 mM-salicylate completely inhibited the phosphate uptake, but part of the phosphorylation accompanying the oxidation of α -oxoglutarate appeared to be resistant to salicylate. The swelling of mitochondria which occurs spontaneously in 0.25 M-sucrose solution was inhibited by salicylate.

METHODS

Preparation of mitochondria. Male Wistar rats (250–300 g.) were killed by stunning, and the livers were removed, weighed and rapidly homogenized in 5–7 vol. of ice-cold 0.25 M-sucrose solution in an all-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min. at 600 g to remove red cells, nuclei and cell debris and the supernatant was centrifuged for 20 min. at 5000 g. The sediment was suspended in 0.25 M-sucrose and centrifuged for 10 min. at 15 000 g and the mitochondrial sediment resuspended in a volume of 0.25 M-sucrose numerically equal (in ml.) to the original weight (in g.) of the liver; thus 1 ml. of this suspension was derived from 1 g. of liver. The nitrogen content of the mitochondrial suspensions varied from 2.0 to 2.9 mg. of N/ml. of suspension. The morphological purity of the preparations was checked by phase-contrast microscopy; occasional nuclei were seen but cell debris was absent.

Measurement of oxidative phosphorylation. Portions (0.5 ml.) of the mitochondrial suspension were added to Warburg flasks containing 0.02 M-potassium phosphate buffer (pH 7.4), 0.01 M-KCl, 5 mM-MgSO₄, mM-sodium adenosine triphosphate (ATP), 0.01 M-glucose, 0.8 mg. of hexokinase [L. Light & Co., Ltd.; activity 28 000 K.M. (Kunitz & McDonald, 1946) units/g.] in 0.2 ml. of 1%

(w/v) glucose solution, 0.1–5.0 mM-sodium salicylate, 0.03 M-NaF and 0.01 M-substrate, in a total volume of 2.0 ml. The substrates used were succinate, β -hydroxybutyrate and α -oxoglutarate as their sodium salts. The α -oxoglutarate solution was prepared and added, together with the fluoride, immediately before the addition of the mitochondrial suspension. Perchloric acid (0.2 ml.; 60%, w/w) was placed in the side arm of each flask and the centre well contained 0.2 ml. of 20% (w/v) KOH solution. The flasks were incubated with shaking at 25°, the gas phase being air. After 6 min. for equilibration, the oxygen uptake was measured for 30 min. and phosphate measured in the reaction mixtures by the method of Allen (1940). Initial values for the phosphate were obtained at zero time and experimental values after incubation for 30 min.; the reactions were stopped by the addition of the perchloric acid from the side arm. The disappearance of inorganic phosphate from the reaction mixture was taken as a measure of the amount of high-energy-bond formation. The oxygen consumption was estimated in a number of experiments with α -oxoglutarate as a substrate and without sodium ATP, to study the effects of salicylate on the respiration of mitochondria in an ATP-deficient system.

Measurement of swelling of mitochondria. Mitochondrial suspension (0.1 ml.) was added to optical cuvettes containing 3.7 ml. of 0.25 M-sucrose and 0.02 M-2-amino-2-hydroxymethylpropane-1:3-diol HCl (tris) buffer, pH 7.4. Either 0.2 ml. of water or 0.2 ml. of sodium salicylate solution, to give final salicylate concentrations from 0.1 to 5.0 mM, was added and the extinctions were measured at 520 m μ in a Hilger Uvispek spectrophotometer against a solution in which 0.1 ml. of 0.25 M-sucrose solution was substituted for the mitochondrial suspension.

RESULTS

Results in Table 1 show that 0.1–5.0 mM-salicylate had no significant effect on the oxygen uptake of the mitochondrial suspension with α -oxoglutarate as substrate ($P > 0.1$ for all salicylate concentrations), but it decreased the uptake of inorganic phosphate. This effect became significant ($P 0.02$) at a concentration of 0.5 mM-salicylate where the mean P/O ratio was approximately two-thirds of the corresponding control value. Table 2 contains the results of similar experiments with β -hydroxybutyrate as substrate. The oxygen consumption was not significantly affected by the salicylate ($P > 0.1$) but 0.1, 0.5 and 1.0–5.0 mM-salicylate caused 16, 80 and 100% inhibition respectively of

the phosphate uptake. The higher salicylate concentrations (1–5 mM) produced an increase of orthophosphate in the reaction mixture.

The results with succinate as substrate (Table 3) resemble those observed with β -hydroxybutyrate except that the higher concentrations of salicylate also produced a depression of the oxygen uptake of the mitochondrial suspensions, amounting to about 40% for 5 mM-salicylate. Comparison of the amounts of orthophosphate present initially (40 μ moles) and those remaining after incubation in the various experiments showed no significant difference ($P > 0.1$) for 5 and 2 mM-salicylate in Table 1 and for 0.5 mM-salicylate in Table 2. In all

other cases where a phosphate uptake was observed the difference was significant ($P < 0.02$).

With an ATP-deficient system and α -oxoglutarate as substrate the mitochondrial suspensions showed a low rate of oxygen uptake (Fig. 1), but in the presence of 5 mM-salicylate the oxygen consumption increased almost to the rate obtained when optimum amounts of ATP (mM) were present.

Fig. 2 shows that 5 mM-salicylate completely prevented the swelling of mitochondria suspended in a solution containing 0.25 M-sucrose and 0.02 M-tris at pH 7.4. This effect was less evident with the lower salicylate concentrations and absent with 0.1 M-salicylate. Pennial (1958) has shown that in

Table 1. *Effect of salicylate on the P/O ratios of mitochondria with α -oxoglutarate as substrate*

Reaction mixture: 0.02 M-potassium phosphate buffer, pH 7.4, 16 mM-KCl, 5 mM-MgSO₄, mM-sodium ATP, 0.01 M-glucose, 0.01 M- α -oxoglutarate, hexokinase (0.8 mg.) in 0.2 ml. of 1% glucose, 0.1–5.0 mM-sodium salicylate, 0.03 M-sodium fluoride and 0.5 ml. of mitochondria in 0.25 M-sucrose, in a total volume of 2.5 ml. The side arm of the Warburg flasks contained 0.2 ml. of 60% perchloric acid and the centre well 0.2 ml. of 20% KOH and filter paper. The nitrogen content of the mitochondrial suspension ranged from 2.0 to 2.9 mg. of N/ml. of suspension. The temperature was 25° and the gas phase was air. Incubation was carried out for 30 min., after a 6 min. equilibration period. Mean values are given for the results.

Concn. of salicylate (mM)	Number of experiments	Uptake of O ₂ (μ g.atoms)	Uptake of P (μ g.atoms)	P/O ratios
0	42	3.78	8.7	2.30
5.0	11	4.27	0.4	0.09
2.0	5	4.11	0.8	0.19
1.0	5	3.99	3.4	0.85
0.5	3	3.95	6.0	1.52
0.1	5	4.01	8.2	2.04

Table 2. *Effect of salicylate on the P/O ratios of mitochondria with β -hydroxybutyrate as substrate*

Experimental conditions were as described in Table 1, with 0.01 M- β -hydroxybutyrate as substrate; results are given as mean values.

Concn. of salicylate (mM)	Number of experiments	Uptake of O ₂ (μ g.atoms)	Uptake of P* (μ g.atoms)	P/O ratios
0	13	2.39	5.1	2.13
5.0	3	2.96	-5.6	—
2.0	3	3.13	-3.3	—
1.0	3	2.39	-0.8	—
0.5	3	2.62	1.0	0.38
0.1	3	3.02	4.3	1.42

* Negative values indicate an increase in the orthophosphate content of the medium.

Table 3. *Effect of salicylate on the P/O ratios of mitochondria with succinate as substrate*

Experimental conditions were as described in Table 1, with 0.01 M-succinate as substrate; mean values are given for the results.

Concn. of salicylate (mM)	Number of experiments	Uptake of O ₂ (μ g.atoms)	Uptake of P* (μ g.atoms)	P/O ratios
0	12	5.16	8.3	1.61
5.0	6	2.82	-4.9	—
2.0	2	4.61	-3.6	—
1.0	3	3.13	-2.3	—
0.5	3	5.33	3.1	0.58
0.1	3	5.42	7.6	1.40

* Negative values indicate an increase in the orthophosphate content of the medium.

hypotonic media 2 mM-salicylate caused an initial slowing of mitochondrial swelling followed by an accelerated rate of swelling in the later stages.

DISCUSSION

These results are in general agreement with those of Brody (1956), Penniall *et al.* (1956) and Penniall (1958) in demonstrating that salicylate uncouples the phosphorylations associated with the oxidation of substrates, such as succinate and β -hydroxybutyrate, in respiring mitochondrial suspensions. The work of Brody (1956) and Penniall (1958), which showed that salicylate resembled other uncoupling agents in stimulating the respiration of mitochondria in an acceptor-deficient system (Lardy & Wellman, 1952), has been confirmed. The zero values for the P/O ratios with succinate and β -hydroxybutyrate in the presence of 1–5 mM-salicylate indicated that these concentrations of salicylate uncouple the phosphorylations associated with the entire respiratory chain. A portion of the phosphorylation accompanying the oxidation of α -oxoglutarate may be resistant to 1 and 2 mM-salicylate (Table 4). Similarly, with lower salicylate concentrations (0.5 and 0.1 mM) the α -oxoglutarate values are higher than with the other two substrates. A possible explanation of this effect is that the substrate phosphorylation accompanying α -oxoglutarate oxidation (Hunter, 1951) is not affected by salicylate. The accumulation of excess of inorganic phosphate in the presence of 1–5 mM-salicylate, with succinate or β -hydroxybutyrate as substrate (Tables 2 and 3), suggests that these concentrations of salicylate may stimulate adeno-

sine triphosphatase, although stimulation of other phosphatases cannot be excluded.

Lehninger (1956) has classified uncoupling agents into two main groups depending on whether they prevent or increase the swelling of mitochondria which occurs in 0.25 M-sucrose solution. The present results show that salicylate resembles the dinitrophenols in belonging to the former group. Lehninger (1956) has suggested that the different effects of uncoupling agents on mitochondrial swelling may be related to their sites of action either at the mitochondrial surface or on intramitochondrial-enzyme systems.

Fig. 2. Effect of salicylate on swelling of mitochondria. Mitochondrial suspension (0.1 ml.) was added to 3.7 ml. of solution containing 0.25 M-sucrose and 0.02 M-tris HCl buffer, pH 7.4; 0.2 ml. of water was added to control cuvettes and 0.2 ml. of salicylate solution (to give final salicylate concentrations from 0.1 to 5.0 mM) to experimental cuvettes. Extinction values were measured at 520 m μ in a Hilger Uvispek spectrophotometer against a solution in which 0.1 ml. of 0.25 M-sucrose solution was substituted for the mitochondrial suspension. ●, 5.0 mM-Salicylate; ×, 1.0 mM-salicylate; ■, 0.5 mM-salicylate; □, 0.1 mM-salicylate; ○, control.

Fig. 1. Effect of salicylate on oxygen uptake of mitochondria in an ATP-deficient system. Reaction mixtures were as described in Table 1. ○, Control; ●, 5 mM-salicylate with ATP omitted from reaction mixture; ×, control with ATP omitted from reaction mixture.

Table 4. Effects of salicylate in uncoupling oxidative-phosphorylation reactions with various substrates

Results are derived from those contained in Tables 1–3.

Concn. of salicylate (mM)	Substrate: β -Hydroxybutyrate		
	α -Oxoglutarate	Succinate	
P/O ratios (expressed as a percentage of the controls)			
5.0	4	0	0
2.0	8	0	0
1.0	37	0	0
0.5	66	18	36
0.1	89	67	87

The possible relation between the uncoupling action of salicylate on oxidative-phosphorylation reactions and other effects of the substance on carbohydrate metabolism in animals has already been discussed by Smith & Jeffrey (1956). Some further examples have been reported. Manchester, Randle & Smith (1958) found that salicylate increased the glucose uptake of rat diaphragm incubated in a bicarbonate medium. This observation is explicable (Randle & Smith, 1958) on the basis that the entry of glucose into the muscle cells is normally restrained by a process dependent on a substance generated during oxidative phosphorylation and that this restraint is diminished by the uncoupling action of salicylate. The incorporation of ^{14}C into the liver glycogen of intact rats after the injection of $[2\text{-}^{14}\text{C}]\text{acetate}$ is inhibited by salicylate (Smith, 1958). This finding is consistent with the view that salicylate impairs carbohydrate synthesis by interfering with oxidative-phosphorylation processes and hence the production of high-energy-phosphate-bond compounds which are necessary at various steps in the major pathway by which acetate carbons are incorporated into liver glycogen.

SUMMARY

1. The effects of salicylate on oxidative-phosphorylation reactions in mitochondria prepared from rat liver have been studied.

2. The phosphorylation associated with the oxidation of succinate and β -hydroxybutyrate was completely uncoupled by 1 mM-salicylate and higher concentrations; 0.1 and 0.5 mM produced smaller but still definite effects.

3. Part of the phosphorylation accompanying

the oxidation of α -oxoglutarate appeared to be resistant to salicylate.

4. 5 mM-Salicylate increased the rate of oxygen consumption in an acceptor-deficient system above that in a corresponding control system saturated with glucose and hexokinase.

5. 5 mM-Salicylate completely prevented the swelling of mitochondria occurring in 0.25M-sucrose solution. Lower salicylate concentrations caused less-marked effects.

6. Some implications of these results are discussed.

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Some Cation-binding Properties of Cartilage

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For some years there has been discussion of the importance of chondroitin sulphate in the calcification of cartilage. It has been suggested (Stetten, 1950; Neuman, 1952) that chondroitin sulphate should behave analogously to ion-exchange resins in the binding of cations.

Boyd & Neuman (1951) have shown that sodium, calcium and barium ions are equally bound to decalcified calf costal cartilage, that the binding capacity is closely related to the chondroitin sulphate content and that the binding of calcium ions is an ion-exchange reaction.

Sobel (1955) has considered the inactivation of the calcification *in vitro* of rachitic-bone sections by cations. The decreasing order of inactivating power was shown to be Be^{2+} , Cu^{2+} , Mg^{2+} , Na^{+} , Sr^{2+} , K^{+} , and this order is in the same direction as the reciprocal of the ionic radii. The mechanism of inactivation is said to involve competition between the ions for the chondroitin sulphate complex present in the organic matrix.

Simkiss & Tyler (1958) have studied the cation-binding properties of the organic matrix of egg shells. An acid mucopolysaccharide has been sug-