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The Effect of Salicylic Acid, Benzoic Acid and some of their Derivatives on Oxidative Phosphorylation

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Salicylic acid (10^{-2} M) and benzoic acid (10^{-1} M) cause an uncoupling of oxidative phosphorylation from respiration in isolated rat liver mitochondria. Similar results are obtained with 5-nitro-salicylic acid and with methyl benzoate.

The growth inhibiting effect of salicylic and benzoic acids on microorganisms has been the subject of many studies with the purpose of elucidating the mode of action of these compounds. In recent years investigations have been published which call attention to the possibility that the growth inhibiting effect might originate, at least to some extent, in an inhibition of the formation or utilization of high energy compounds [1-5]. In order to obtain further evidence, the effect of salicylic and benzoic acids and some of their derivatives on the oxidative phosphorylation in isolated rat liver mitochondria was studied in this work.

EXPERIMENTAL

Preparation of mitochondria. The preparation of mitochondria was carried out according to the method of Schneider and Hogeboom [6]. The rats were killed by decapitation and the livers put in 0.25 M ice-cold sucrose as fast as possible. In this solution the liver was cut into small pieces and rinsed several times with sucrose solution. The homogenization was carried out in a Potter-Elvehjem homogenizer. The homogenate was diluted to *ca.* 100 ml with 0.25 M sucrose and centrifuged at 1 600 *g* for 10 minutes. The supernatant was decanted and recentrifuged at 4 100 g for 20 min. The mitochondria were washed twice with *ca.* 100 ml sucrose each time.

Materials. Yeast hexokinase was used as a stock solution which had been prepared according to Berger *et al.*[7] with minor modifications [8]. ATP, as the crystalline disodium trihydrate, had been furnished by the Sigma Chemical Company, St. Louis, Mo., U.S.A., and $H_3{}^{32}PO_4$ by the Radiochemical Centre, Amersham, England. All solutions were prepared in glass-distilled deionized water.

Incubation procedure. Mitochondria from one liver were suspended in 13.3 ml 0.25 M sucrose containing 116 mg $K_2H^{32}PO_4$, 144 mg glucose and 12 mg ATP. The pH was adjusted to 7.5 by means of glass electrode. To this suspension 1.3 ml 0.1 M $MgCl_2$ was added and the suspension was diluted to 20 ml with 0.25 M sucrose. In all experiments 1.5 ml of this suspension was added to each vessel.

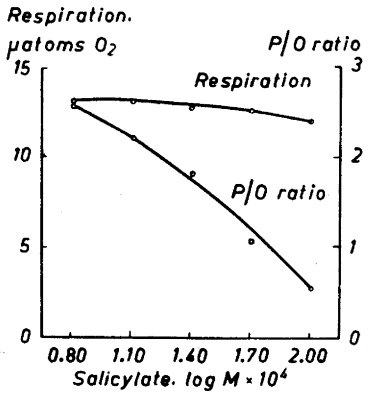

Fig. 1. The effect of increasing concentrations of salicylate on oxidative phosphorylation. Each vessel contained besides the suspension described in the experimental part 30 μmoles potassium α-ketoglutarate as substrate and potassium salicylate in the concentrations indicated in the figure. Experimental conditions: gas phase, air; temp., 30°C; time of incubation, 20 min.

Analytical procedures. Oxygen consumption was measured by the conventional Warburg technique. The described suspension and neutralized solutions of the different inhibitors were added to the main compartments of the Warburg vessels, substrates and hexokinase (0.1 ml of the stock solution) to the sidearms. The centre wells contained 0.2 ml 2 M KOH, absorbed on a roll of filter paper. The final volume of solution in the vessels was adjusted to 2 ml with 1.15 % KCl. Manometer readings were made at 5 min invervals, starting 5 min after the addition of substrate and hexokinase from the sidearms. Oxygen consumption for the period 0—5 min was calculated by extrapolation. At the end of the experiments, enzymatic activity was terminated by the addition of 0.2 ml 5 M H_2SO_4. Phosphate uptake was determined as the ratio of organic phosphate formed to inorganic phosphate added by the isotope distribution method described by Lindberg and Ernster [9].

RESULTS

The effect of increasing concentrations of salicylate on oxidative phosphorylation and oxygen consumption with α-ketoglutarate as substrate is illustrated by Fig. 1.

The figure shows that salicylate at a concentration of about 1×10^{-2} M almost completely inhibits phosphorylation, while the inhibition of oxygen consumption is negligible. Similar results are obtained if α-ketoglutarate is replaced by pyruvate + malate, succinate, or glutamate.

Table 1. The effect of salicylate on oxidative phosphorylation.

Additions	Without hexokinase	With hexokinase	
	Oxygen μatoms	Oxygen μatoms	P/O ratio
None	4.6	11.2	2.5
DPN + cytochrome c	5.3	11.7	2.3
Salicylate	9.4	8.5	0.6
Salicylate + DPN + cytochrome c	10.3	9.6	0.5

The final concentrations of the substances added to the suspension of the mitochondria were as follows: DPN, 6.8×10^{-4} M; cytochrome c, 10^{-5} M; potassium salicylate, 10^{-2} M, Each vessel contained 30 μmoles potassium α-ketoglutarate as substrate. Experimental conditions as in Fig. 1.

Table 1 exemplifies an experiment which shows that salicylate, at the concentration mentioned above, is able to induce an almost maximum level of respiration in the absence of hexokinase. This is a further indication that salicylate uncouples phosphorylation from respiration, since, in this case, respiration proceeds in the absence of a terminal phosphate acceptor.

Experiments with benzoate showed that this substance had a similar effect, which, however, became noticeable only at still higher concentrations than those which were necessary in the case of salicylate. The results of a typical experiment with benzoate are shown in Table 2. The relatively high concentrations of benzoate caused a partial inhibition of respiration along with the effect on phosphorylation. It was possible to bring up the respiration to the original level by the addition of DPN and cytochrome c, without, however, a simultaneous recovery of the phosphorylation. This indicates that the inhibition of respiration is a secondary effect, due to a release of DPN and cytochrome c from the mitochondria.

Similar pictures are obtained if glutamate is replaced by other substrates such as succinate and α-ketoglutarate.

Table 2. The effect of benzoate on oxidative phosphorylation.

Additions	Without hexokinase	With hexokinase	
	Oxygen μatoms	Oxygen μatoms	P/O ratio
None	3.5	12.3	2.1
DPN + cytochrome c	6.7	12.2	2.0
Benzoate	8.2	10.7	1.0
Benzoate + DPN + cytochrome c	8.7	12.1	0.9

Experimental conditions as in Fig. 1. Each vessel contained 30 μmoles potassium glutamate. DPN and cytochrome c were added at the same concentrations as in Table 1. The benzoate concentration was 8×10^{-2} M.

In order to further elucidate the mode of action of salicylate and benzoate on oxidative phosphorylation, the effect of some related substances was investigated. Of these compounds 5-nitro-salicylic acid (which differs from 2,4-dinitrophenol only by the replacement of one of the nitro groups by a

Table 3. The effect of 5-nitro-salicylic acid on oxidative phosphorylation.

Substrate	Without 5-nitro-salicylic acid		With 10^{-3} M 5-nitro-salicylic acid		With 10^{-2} M 5-nitro-salicylic acid	
	Oxygen μatoms	P/O ratio	Oxygen μatoms	P/O ratio	Oxygen μatoms	P/O ratio
Glutamate	13.4	2.3	12.8	1.8	11.1	0.14
Succinate	13.8	1.5	13.9	1.2	4.8	0.15

Each vessel contained 30 μmoles substrate. Experimental conditions as in Fig. 1.

carboxyl group) and methyl benzoate inhibited phosphorylation to about the same extent as did salicylic acid, while 4-hydroxy-salicylic, 5-hydroxy-salicylic and 4-amino-salicylic acids (PAS) were without effect. Table 3 shows the results of an experiment with 5-nitro-salicylic acid.

DISCUSSION

It is evident from the results described in this paper that salicylic and benzoic acids produce an effect on oxidative phosphorylation, which is reminiscent of that obtained with 2,4-dinitrophenol. The concentrations required to obtain a marked effect are of the same magnitude as those required for a complete suppression of bacterial growth at the pH in question (7.5). As is well known, however, the growth inhibiting effect of salicylic and benzoic acids increases as the pH is lowered. This is probably due to the fact that the undissociated molecules of these acids are more lipophilic than the respective anions and, thus, more easily taken up by the bacteria. Such a correlation is well conceivable to prevail even in the present case, where an increasing lipid solubility might facilitate the penetration of these inhibitors into the mitochondrial structure. This point is, however, hard to test in experiments with isolated mitochondria, which lack the mechanism by which bacterial cells can make themselves independent at least to some extent of the pH of the environment. A lowering of the pH in such experiments may thus render oxidative phosphorylation impossible.

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