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Inhibition of the mitochondrial oxidation of octanoate by salicylic acid and related compounds

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The inhibition of mitochondrial octanoate oxidation *in vitro* by salicylic acid (1mM), 2,4-dinitrophenol (0·05mM) and γ-resorcylic acid (2mM) was studied. Concentrations of salicylate and 2,4-dinitrophenol markedly inhibiting the mitochondrial oxidation of octanoate did not significantly affect the mitochondrial oxidation of octanoylcarnitine. All three compounds (0·4mM) inhibited a partially purified preparation of medium chain-length acyl-CoA synthetase from ox liver mitochondria. It is suggested that at least part of the inhibition of mitochondrial oxidation by salicylic and γ-resorcylic acids *in vitro* in the concentration ranges studied is caused by the direct inhibition of the medium chain length acyl-CoA synthetase.

Salicylic acid has been reported to produce a number of effects on fatty acid metabolism, both *in vivo* and *in vitro*, including inhibition of fatty acid biosynthesis (Goldman, 1967) and of free fatty acid mobilization (Bizzi, Garattini & Veneroni, 1965). It also inhibits the mitochondrial oxidation *in vitro* of medium chain-length fatty acids (Brody, 1956).

Judah & Rees (1953) have presented evidence that the inhibition of the mitochondrial oxidation of octanoate by 2,4-dinitrophenol, a compound which, like salicylate (Brody, 1956), is an uncoupler of oxidative phosphorylation, is associated with its inhibition of ATP synthesis via its uncoupling action, but no comparable study appears to have been made with salicylate.

This paper presents some results obtained in a comparison of the inhibitory properties towards medium chain-length fatty acid oxidation of salicylate and 2,4-dinitrophenol, including also a structural analogue of salicylate, γ-resorcylic acid (2,6-dihydroxybenzoic acid), which, however, does not uncouple oxidative phosphorylation (Brody, 1956; Whitehouse & Dean, 1965).

MATERIALS AND METHODS

Materials. Salicylic, palmitic, sorbic and γ-resorcylic acids and 2,4-dinitrophenol were obtained from BDH Chemicals Ltd., Poole, U.K., the latter three compounds being purified by recrystallization before use; octanoyl chloride from Aldrich Chemical Co. Inc., Milwaukee, U.S.A.; mercaptoethanol from Sigma (London) Chemical Co. Ltd., London; and DL-carnitine hydrochloride from Calbiochem Ltd., London. DL-Octanoylcarnitine was synthesized from octanoyl chloride and DL-carnitine hydrochloride (Bremer, 1962). Other materials were as described elsewhere (Graham & Park, 1969). The pH of reagent solutions was adjusted with KOH or HCl.

Mitochondrial oxidation of fatty acid. Mitochondria from the livers of rats were freshly prepared for each set of experiments essentially by the method of Hogeboom

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(1955), the mitochondria being finally suspended in a medium composed of 50 mM tris-chloride buffer, 100 mM KCl and 5 mM $MgCl_2$ adjusted to pH 7·4 (Hird & Symons, 1962). The final suspension contained mitochondria from 0·7–1·0 g liver in 1 ml.

The final incubation medium, unless otherwise stated, contained (mM): tris-chloride buffer 32·5 (pH 7·4); KCl, 105; KH_2PO_4, 2·5; AMP, 1·25; ATP, 0·5; DL-carnitine, 0·5; $MgCl_2$, 1·25; fatty acid, 1·0; and 0·5 ml mitochondrial suspension (containing 6–15 mg protein) in a total volume of 2 ml. The pH of the stock solutions of these components had been adjusted to 7·4.

Reaction was initiated by addition of mitochondrial suspension, and took place over 30 min at 25° in either a Warburg respirometer or in wide diameter test tubes with continuous agitation (Lehninger, 1955). After incubation, the reaction mixtures were deproteinized by cooling on ice and adding 2 ml 10% trichloroacetic acid. Acetoacetate was then determined by the method of Barkulis & Lehninger (1951).

Mitochondrial oxidation of octanoylcarnitine. This was as described above, except that no octanoate, DL-carnitine or ATP was included in the incubation medium and DL-octanoylcarnitine was present at a concentration of 1·25 mM.

Determination of protein. Protein in mitochondrial fractions was determined according to Layne (1957) and in other solutions by the method of Miller (1959), using incubation for 30 min at room temperature (20°) instead of at 50°. Bovine serum albumin was used as standard in both methods.

Partial purification and determination of activity of medium chain-length fatty acyl-CoA synthetase. The enzyme [Acid: CoA ligase (AMP), EC 6.2.1.2] was purified from fresh ox liver essentially by the method of Mahler, Wakil & Bock (1953) to the stage before the treatment with alumina Cγ. Solutions used in the extraction and fractionation stages contained 10 mM mercaptoethanol. The enzyme was kept as a concentrated solution in $KHCO_3$ (20 mM)-mercaptoethanol (10 mM) at 1°.

Enzymic activity was determined at 25° by a direct reading assay based on the procedure of Wakil & Hübscher (1960) in which the conversion of sorbic acid into sorboyl-CoA was followed by measuring the change in extinction at 300 nm. The reaction medium, unless otherwise stated, was composed of (mM): tris-HCl, pH 8·0, 50; mercaptoethanol 10; $MgCl_2$, 0·5; ATP, 0·5; CoA, 0·036; sorbic acid, 0·5; and enzyme (0·02 ml containing 428 μg protein) in a total volume of 1 ml. Extinctions were measured over 3–10 min in a 1 cm path-length cell, linear traces of extinction against time being obtained. The molar extinction coefficient of CoA at 300 nm under these conditions was taken to be 19 300 litre mol^{-1} cm^{-1} (Wakil & Hübscher, 1960).

Determination of CoA. CoA was determined under conditions similar to those of the enzyme assay but with the following alterations to the substrate and enzyme concentrations (mM): $MgCl_2$, 12·5; ATP, 25–27; sorbic acid, 10; enzyme protein, 1–2 mg ml^{-1}; CoA, approx. 0·02. Constant values of extinction were obtained in 20–45 min at 25°.

RESULTS

Mitochondrial oxidation

Initial observations confirmed the inhibition of the mitochondrial oxidation of octanoate by salicylate (63% inhibition of acetoacetate production by 1·0 mM concentration) and by 2,4-dinitrophenol (90% inhibition by 0·05 mM) and indicated that γ-resorcylate also acted as an inhibitor (53% inhibition by 2·0 mM). Double reciprocal plots of measurements of rates of acetoacetate production at different octanoate

concentrations in the presence and absence of salicylate (2 mM), 2,4-dinitrophenol (0·025 mM) and γ-resorcylate (2 mM) showed apparently non-competitive, uncompetitive and non-competitive kinetics respectively.

Increasing the concentration of ATP in the reaction medium did not reverse the inhibition by 1 mM salicylate. On the contrary, raising both the ATP and AMP concentrations to 4·0 mM caused 1 mM salicylate to inhibit completely acetoacetate production.

Experiments with the long chain-length fatty acid palmitic acid indicated that oxidation of this compound to acetoacetate was also inhibited by salicylate (55% inhibition by 2 mM).

Mitochondrial oxidation of octanoylcarnitine

To determine whether the above inhibition was occurring at the initial activation stage, the effect of the inhibitors on the oxidation of octanoylcarnitine was examined. This compound could by-pass the acyl-CoA synthetase reaction by the mechanism (Bremer, 1962), octanoyl-carnitine + CoA → octanoyl-CoA + carnitine, and the effect of the inhibitors should be eliminated if they acted only on the activation stage and had no effect on the above reaction or on the later stages of fatty acid oxidation.

Salicylic acid (1 mM) and 2,4-dinitrophenol (0·025 mM) had negligible effects on both acetoacetate formation and oxygen consumption. This compares with the marked effect these concentrations of inhibitor would have had on the mitochondrial oxidation of octanoate itself.

With γ-resorcylic acid, a 2 mM concentration produced an inhibition of 20%; this corresponds to a value of 53% obtained in the oxidation of octanoate; with a 5 mM concentration, inhibition of acetoacetate formation had increased to only 41%.

Inhibition of medium chain-length fatty acyl-CoA synthetase

The inhibition of the partially purified enzyme by the three compounds was determined by measuring the rate of conversion of sorbic acid into sorboyl-CoA. In all cases 0·4 mM concentrations produced an inhibition of 50 to 60% (Table 1).

Table 1. *Inhibition of medium chain-length acyl-CoA synthetase by salicylic acid, 2,4-dinitrophenol and γ-resorcylic acid.* Rates are quoted as mean values ± the experimentally observed range of duplicate determinations.

Inhibitor present	Inhibitor concn (mM)	Rate of formation of sorboyl-CoA (μM min^{-1})	Inhibition (%)
—	—	0·83 ± 0·01	—
Salicylic acid	0·41	0·38 ± 0·00	54
	0·82	0·05 ± 0·01	94
2,4-Dinitrophenol	0·40	0·33 ± 0·01	60
	0·81	0·18 ± 0·00	78
γ-Resorcylic acid	0·40	0·40 ± 0·00	52
	0·80	0·11 ± 0·01	87

Although medium chain-length acyl-CoA synthetase does not catalyse the conversion of salicylic acid into salicyloyl-CoA (Schachter & Taggart, 1954; Killenberg, Davidson & Webster, 1971), the activity of the enzyme fraction used towards salicylate was checked by assaying the CoA after incubation with salicylate. The concentrations of reactants present in the incubation mixture were (mM): $MgCl_2$, 4·9; ATP, 4·9; CoA, 0·22; salicylate, 0·20; and enzyme protein, 990 μg ml^{-1}. Duplicate

tubes along with controls with concentrations as above less salicylate were incubated at 25° for 30 min and 0·1 ml aliquots from each tube assayed for CoA. The mean changes in extinction at 300 nm in the absence of salicylate were 0·39 ± 0·005, and in its presence 0·39 ± 0·004. These results indicate that the extent of the conversion of salicylate into the CoA derivative is not significant.

DISCUSSION

Comparison of the effects of the inhibitors on mitochondrial octanoate oxidation with those on octanoylcarnitine oxidation tends to support the view that the action of all three compounds in the concentrations studied is primarily on the fatty acid activation stage, although the situation is less definite in the case of γ-resorcylate than with the other two inhibitors.

The observation of the inhibition of the medium chain-length fatty acyl-CoA synthetase by all three compounds would suggest at least a partial role for that mechanism in the inhibition of the mitochondrial oxidation in the case of salicylate and γ-resorcylate. With 2,4-dinitrophenol, the concentrations inhibiting the purified enzyme are greater by a factor of ten than those inhibiting mitochondrial octanoate oxidation. In this case, therefore, the predominant effect seems to be by other than the direct inhibition, presumably by an uncoupling action.

The lack of catalytic activity of the enzyme fraction towards salicylic acid as substrate would appear to eliminate this as a mechanism of apparent inhibition. It is unlikely that the enzyme would show catalytic activity towards γ-resorcylic acid and 2,4-dinitrophenol in the absence of any such activity towards salicylate.

Preliminary investigations of the mechanism of inhibition of the synthetase by these compounds (Park, unpublished results) have indicated a more complex behaviour of the enzyme at the lower protein and CoA concentrations used here than observed previously (Graham & Park, 1969), non-linear (convex upwards) double reciprocal plots being obtained both in the presence and absence of the inhibitors with ATP or sorbate as the substrate whose concentration is varied.

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