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EFFECTS OF SALICYLATE, γ-RESORCYLATE AND GENTISATE ON OXIDASE ENZYME SYSTEMS FROM GUINEA-PIG LIVER MITOCHONDRIA

W. J. W. HINES, C. BRYANT and M. J. H. SMITH

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Empire Rheumatism Council Research Unit, Chemical Pathology Department, King's College Hospital Medical School, London

Abstract—The effects of salicylate, γ-resorcylate and gentisate on the oxidative activity of a particulate preparation from guinea-pig liver were studied. Low concentrations (0·1 mM) of gentisate markedly inhibited the succinate oxidase and succinate–cytochrome c reductase activities of the preparation. Considerably higher concentrations of salicylate and resorcylate were required to produce similar inhibitions of these enzyme systems. In high concentrations (50 mM) the three drugs inhibited succinate dehydrogenase and cytochrome oxidase activities. It was concluded that gentisate exerted a specific effect at some point on the electron chain between succinate dehydrogenase and cytochrome c. The high concentration of salicylate and resorcylate required for inhibition indicated a non-specific, "blanket" effect on several of the steps in the respiratory chain. Salicylate (10 mM), resorcylate (1 mM) and gentisate (0·1 mM) also inhibited the NADH-cytochrome c reductase activity of the preparation. The inhibitions due to salicylate and resorcylate, but not that caused by gentisate, were completely reversed by the addition of higher concentrations of NADH.

SALICYLATE concentrations, in the range 5 to 7 mM ,have been reported to interfere with the oxidation of succinate in homogenates of rat tissues[1] and in respiring mitochondrial preparations.[2] However, it has been shown[3] that salicylate concentrations in this range inhibit malate dehydrogenase and an apparent effect of salicylate on succinate oxidation could have resulted from the drug primarily affecting the further metabolism of malate. Lutwak-Mann[4] found that very high concentrations (> 50 mM) of salicylate inhibited both the succinate oxidase system and succinate dehydrogenase [succinate: (acceptor) oxidoreductase, E.C. 1.3.99.1.] in rat skeletal muscle. This paper is concerned with the inhibitory effects of 10 to 200 mM salicylate on several components of the succinate oxidase system and also with a comparison of the actions of salicylate with two of its congeners, γ-resorcylate (2:6-dihydroxybenzoate) and gentisate (2:5-dihydroxybenzoate).

METHODS

Materials

NADH and cytochrome c were obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany, phenazine methosulphate from Aldrich Chemical Co., Inc., Milwaukee, Wis., U.S.A., palladised (5% Pd) asbestos from British Drug Houses Ltd., Poole, Dorset, and [1:4-$^{14}C_2$] succinic acid (specific activity, 1 μc per 0·4 μmole) from the Radiochemical Centre, Amersham, Bucks. Reduced cytochrome c

was prepared by the method of Smith.[5] The γ-resorcylic and gentisic acids were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. and recrystallised from aqueous ethanol until their melting points remained constant. These congeners, as well as A.R. salicylic acid, were dissolved in 0·1 M potassium phosphate buffer, pH 7·4 to give solutions which if necessary were adjusted to pH 7·4 by the addition of KOH solution before subsequent addition to the various reaction mixtures.

Succinate oxidase preparation

Male albino guinea-pigs (wt. 400–700 g), maintained on a mixed diet, were killed by cervical fracture. The livers from 30 animals were removed and homogenised with 2 l. 0·25 M sucrose (approximately five times the volume of the combined livers) in a Waring blendor. The mitochondrial fraction (approximately 100 ml) was separated by the method of Schneider and Hogeboom,[6] homogenised in 500 ml distilled water at 4° and allowed to stand with stirring at 4° for 30 min. After centrifugation, in fifty equal portions, at 105,000 × *g* for 30 min, in a Spinco preparative ultracentrifuge, the supernatants were discarded and the lysed mitochondrial pellets stored individually at −40° until use. One pellet homogenised in 20 ml 0·1 M potassium phosphate buffer, pH 7·4, was used as the enzyme preparation. The succinate oxidase activity of the preparation was stable for at least 3 months when stored at −40°. Spectroscopic examination of the enzyme preparation was carried out with a Beck low-dispersion microspectroscope and a 150 c.p. "Pointolite" bulb contained in a Universal lamp (W. Watson and Sons Ltd., Holborn, London, W.C.1). Samples (50 μl) of the enzyme preparation were mixed with 25 μl of 0·1 M potassium phosphate buffer, pH 7·4, containing 0·5 μc (0·2 μmole) of [1:4-$^{14}C_2$] succinic acid and incubated at 37° for 60 min. The radioactive substances in the incubation mixtures were separated by two-dimensional paper chromatography and visualised by radioautography.[7]

Enzyme assays

Succinate oxidase activity was estimated manometrically according to the directions given by Umbreit *et al.*[8] Succinic dehydrogenase activity was measured manometrically by the phenazine methosulphate method.[9] Succinate–cytochrome c reductase, NADH–cytochrome c reductase and cytochrome oxidase activities were measured spectrophotometrically. The details of the reaction systems are given in the tables.

RESULTS

Characterisation of the enzyme preparation

Direct visual spectroscopic examination of the enzyme preparation showed the presence of absorption bands characteristic of cytochromes a, b and c. Radioactivity, from the labelled succinate, was found to be incorporated into fumarate and malate only, indicating that the enzyme preparation possessed succinate dehydrogenase and fumarase activities but did not contain appreciable amounts of other enzyme activities concerned with the further metabolism of malate.

Succinate oxidase system

The addition of cytochrome c to the enzyme preparation increased the activity of the succinate oxidase system. A maximal effect was observed when 1 mg of the cytochrome was added to 1 ml of the enzyme preparation and this amount was added in

TABLE 1. EFFECTS OF SALICYLATE, RESORCYLATE AND GENTISATE ON SUCCINATE OXIDASE SYSTEM

Each Warburg flask contained 1 ml of the enzyme preparation plus 1 mg of cytochrome c and 1 ml of either 0·1 M potassium phosphate buffer, pH 7·4, or a solution of the drug in the buffer; 0·1 ml 20% (w/v) KOH solution was placed in the centre well and 0·9 ml 0·3 M sodium succinate, dissolved in the buffer, in the side arm. The flasks were incubated at 37° and equilibrated for 30 min before the succinate was added from the side arm by tipping. The results were calculated from the initial rates of oxygen uptake and are expressed as percentage inhibitions of the corresponding control rate. The mean oxygen uptake of the control flasks was 325 μl over a period of 30 min. Each observation represents the mean of six estimations.

Salicylate concn. (mM)	% Inhibition	Resorcylate concn. (mM)	% Inhibition	Gentisate concn. (mM)	% Inhibition
5	0	1	0	0·05	0
10	20	5	30	0·1	70
20	35	10	50	1	100
50	54	20	65	10	100
100	75	50	100		
200	100				

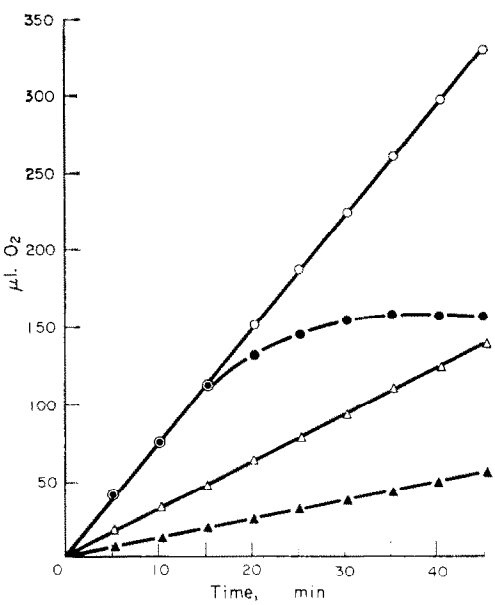

FIG. 1. Effects of salicylate, resorcylate and gentisate on succinate oxidase activity. Each Warburg flask contained 1 ml of the enzyme preparation, and 1 ml of either 0·1 M potassium phosphate buffer, pH 7·4, or a solution of drug in the buffer; 0·1 ml 20% (w/v) KOH solution was placed in the centre well and 0·9 ml 0·2 M sodium succinate, dissolved in the phosphate buffer, in the side arm. The flasks were incubated at 37° and equilibrated for 5 min before the succinate was added from the side arm by tipping. ○, control; ●, 1 mM gentisate; △, 100 mM salicylate; ▲, 40 mM resorcylate.

all subsequent experiments except those in which succinate dehydrogenase activity was being estimated.

The succinate oxidase activity of the enzyme preparation was inhibited by salicylate, γ-resorcylate and gentisate (Table 1). Gentisate was found to be the most powerful inhibitor, being approximately 100 times more active than γ-resorcylate and 500 times more active than salicylate. In the above experiments the enzyme preparation was pre-incubated with the drugs for 30 min before the reaction was started by the addition of succinate. Figure 1 shows that the inhibitory effects of 100 mM salicylate, 40 mM resorcylate were evident immediately after their addition to the reaction mixtures whereas 1 mM gentisate did not completely inhibit the succinate oxidase activity until at least 30 min had elapsed.

Succinate dehydrogenase

Table 2 shows that the succinate dehydrogenase activity of the enzyme preparation is much less sensitive to the inhibitory effects of salicylate, resorcylate and gentisate

TABLE 2. EFFECTS OF SALICYLATE, RESORCYLATE AND GENTISATE ON SUCCINATE DEHYDROGENASE ACTIVITY

Each Warburg flask contained 1 ml of the enzyme preparation and 0·8 ml of either 0·1 M potassium phosphate buffer, pH 7·4, or a solution of the drug in the buffer; 0·3 ml of 1% (w/v) phenazine methosulphate and 0·6 ml 0·4 M sodium succinate, dissolved in the phosphate buffer, were placed in the side arm. Immediately before placing the flask on the manometer, 0·3 ml 0·01 M HCN[9] in distilled water was added to the main compartment of the flask. The vessels were incubated at 37° and equilibrated for 30 min before the succinate was added from the side arm by tipping. The oxygen uptake was measured at 5 min intervals over a period of 30 min. The results were calculated from the initial rates of oxygen uptake and are expressed as percentage inhibitions of the corresponding control rate. Each observation represents the mean of six estimations.

Salicylate concn. (mM)	% Inhibition	Resorcylate concn. (mM)	% Inhibition	Gentisate concn. (mM)	% Inhibition
50	0	20	0	50	0
100	0	50	61	100	67
150	86	100	100	200	100
200	100				

than is the succinate oxidase system. Inhibition only occurred at concentrations in the range 50 to 150 mM for all three drugs whereas 10 mM salicylate and resorcylate and 0·1 mM gentisate inhibited the succinate oxidase system by at least 20 per cent.

Succinate–cytochrome c reductase system

Table 3 shows that salicylate, resorcylate and gentisate inhibited the succinate–cytochrome c reductase system. Gentisate was found to be the most powerful inhibitor causing an approximately 50 per cent effect at 0·1 mM.

NADH–cytochrome c reductase system

The three drugs also inhibited the NADH–cytochrome c reductase system in the enzyme preparation (Table 4) at similar concentrations to those affecting the succinate–cytochorome c reductase system. The results in Table 5 show that the inhibitory

TABLE 3. EFFECTS OF SALICYLATE, RESORCYLATE AND GENTISATE ON SUCCINATE–CYTOCHROME C REDUCTASE SYSTEM

Reaction mixtures consisted of 2·0 ml 0·1 M potassium phosphate buffer, or a solution of the drug in buffer, 0·1 ml 0·2 M sodium succinate, 0·2 ml 0·01 M HCN, 0·1 ml 0·2 mM cytochrome c and 0·3 ml of the enzyme preparation. Measurements were made of the change of E_{550} in a Hilger Uvispek spectrophotometer, at 10 sec intervals over a period of 2 min at room temperature (20 ± 2°), in a glass cell of 1 cm light path, and were used to calculate the initial rates. Each observation is the mean of six estimations.

Salicylate concn. (mM)	% Inhibition	Resorcylate concn. (mM)	% Inhibition	Gentisate concn. (mM)	% Inhibition
20	18	5	0	0·1	45
50	50	10	21	1·0	55
100	70	20	59	10	70
200	100	50	100	50	100

TABLE 4. EFFECTS OF SALICYLATE, RESORCYLATE AND GENTISATE ON NADH–CYTOCHROME C REDUCTASE SYSTEM

Reaction mixtures consisted of 2·0 ml 0·1 M potassium phosphate buffer, pH 7·4, or a solution of the drug in buffer, 0·1 ml 1·25 mM NADH, 0·2 ml of 0·01 M HCN, 0·1 ml 0·2 mM cytochrome c, and 0·01 ml of the enzyme preparation. Measurements were made of the change of E_{550} in a Hilger Uvispek spectrophotometer, at 10 sec intervals over a period of 2 min at room temperature (20 ± 2°), in a glass cell of 1 cm light path, and were used to calculate the initial rates. Each observation is the mean of six estimations.

Salicylate concn. (mM)	% Inhibition	Resorcylate concn. (mM)	% Inhibition	Gentisate concn. (mM)	% Inhibition
10	10	1	12	0·1	25
20	17	5	28	1·0	40
50	45	10	42	10	63
100	80	20	65	50	78
200	100	50	88	100	83

TABLE 5. EFFECT OF CONCENTRATION OF NADH ON INHIBITION OF NADH–CYTOCHROME C REDUCTASE BY SALICYLATE, RESORCYLATE AND GENTISATE

Experimental details as in Table 4.

Concn. of NADH (mM)	% Inhibition 50 mM Gentisate	100 mM Salicylate	10 mM Resorcylate
5·0	70	—	—
2·5	74	0	0
0·50	—	—	10
0·25	73	23	—
0·125	75	66	36
0·050	78	80	42

effect of salicylate and resorcylate were completely removed by increasing the concentration of NADH in the reaction mixtures. However, the inhibition caused by gentisate was not appreciably affected by varying the NADH concentration from 0·05 to 5 mM.

Cytochrome c oxidase

The oxidation of reduced cytochrome c by the enzyme preparation was inhibited by salicylate, resorcylate and gentisate at concentrations greater than 10 mM (Table 6). The three drugs produced similar effects over the concentration ranges used.

TABLE 6. THE EFFECTS OF SALICYLATE, RESORCYLATE AND GENTISATE ON CYTOCHROME OXIDASE ACTIVITY

Reaction mixtures contained 2·3 ml 0·1 M potassium phosphate buffer, pH 7·0, or a solution of the drug in the buffer, 0·2 ml 90μM reduced cytochrome c and 0·01 ml of the enzyme preparation which was added to start the reaction. Measurements were made of the change of E_{550} in a Hilger spectrophotometer, at 10 sec intervals over a period of 2 min at room temperature (20 ± 2°), in a glass cell of 1 cm light path and were used to calculate the initial rates. Each observation is the mean of six estimations.

Salicylate concn. (mM)	% Inhibition	Resorcylate concn. (mM)	% Inhibition	Gentisate concn. (mM)	% Inhibition
10	0	1	0	0·1	0
20	15	5	0	1·0	0
50	41	10	13	10	16
100	53	20	33	50	52
200	88	50	47	100	72

DISCUSSION

The enzyme preparation from guinea-pig liver which was used in the present work contained cytochromes a, b and c, but the further addition of cytochrome c stimulated the activity of the succinate oxidase system. The succinate oxidase activity of the preparation was stable for at least 3 months when stored at −40°. Succinate dehydrogenase, the succinate–cytochrome c reductase system, NADH–cytochrome c reductase, cytochrome oxidase and fumarase were present but enzyme systems capable of the further metabolism of malate were not detected.

Salicylate, γ-resorcylate and gentisate were found to inhibit the succinate oxidase system at several points. Succinate dehydrogenase activity in the enzyme preparation from guinea-pig liver was inhibited by the three compounds at concentrations ranging from 50 to 150 mM, resorcylate being the most active inhibitor and salicylate the least active. Similar results with salicylate were reported by Lutwak-Mann[4] using a minced skeletal muscle preparation from the rat. The complete oxidase system in the guinea-pig liver preparation was much more sensitive to salicylate, γ-resorcylate and gentisate than the succinate dehydrogenase suggesting that the drugs also interfered with the electron transport chain. Gentisate was found to be the most powerful inhibitor of the succinate oxidase system, causing a 70 per cent inhibition at a concentration of 0·1 mM whereas salicylate and γ-resorcylate only produced measurable effects at concentrations of 10 and 5 mM respectively. These results agree with the findings of other workers,[1, 3] that 6·7 mM concentrations of the drugs inhibit the oxidation of succinate

by homogenates of rat kidney cortex and that resorcylate was a more potent inhibitor than salicylate of a sheep-heart succinate oxidase preparation.

In the experiments described in Table 1 the enzyme preparation was preincubated with the drugs before the reaction was started by the addition of substrate. The results in Fig. 1 show that the inhibitory effects of 100 mM salicylate and 40 mM resorcylate on succinate oxidase activity were evident immediately after their addition to the reaction mixtures but that 1 mM gentisate did not completely inhibit until at least 30 min had elapsed. Preincubation of the enzyme preparation with 1 mM gentisate was therefore necessary before maximum inhibition occurred, suggesting that the actual inhibitor was not gentisate itself but a derivative formed during the preincubation. It has been shown[10] that quinones are powerful inhibitors of succinate oxidase activity and that tissue preparations containing cytochrome c and cytochrome oxidase will oxidise phenolic compounds. Gentisate is readily oxidised to its corresponding quinone because the two hydroxyl groups are in the *para* positions on the benzene ring, whereas neither salicylate nor γ-resorcylate are capable of being converted to similar products. Thus the inhibition of succinate oxidase activity by low concentrations of gentisate may result from the formation of 2-carboxy 1:4-benzoquinone during preincubation.

The site of the inhibitory action of low concentrations (0·1 and 1 mM) of gentisate must lie between the succinate dehydrogenase flavoprotein and cytochrome c because these concentrations of gentisate inhibit the complete succinate oxidase system, succinate–cytochrome c reductase and NADH–cytochrome c reductase but do not affect either succinate dehydrogenase or cytochrome oxidase. Attempts were made to define the actual site of inhibition more precisely by using either methylene blue as a hydrogen acceptor or *p*-phenylene diamine as a hydrogen donor. However, the results were not valid because gentisate reacted with both substances, causing visible colour changes.

In addition to the specific inhibitory effect of low gentisate concentrations on a component of the electron chain lying between succinate dehydrogenase and cytochrome c, the drug also inhibited cytochrome oxidase in concentrations of 10 mM and above. It therefore resembles salicylate and resorcylate in its action on one or more components of the electron chain lying between cytochrome c and oxygen (Table 6). Salicylate and resorcylate also inhibited succinate–cytochrome c reductase (Table 4) in concentrations above 10 mM and all three drugs inhibited the succinate dehydrogenase (Table 2) at concentrations ranging from 50 to 150 mM. Thus, salicylate, resorcylate and gentisate in concentrations of 10 mM and above appear to exert a non-specific, "blanket" effect on several constituents of the succinate oxidase system. These inhibitory actions may result from the drugs affecting the colloidal structure of the particulate enzyme system, rather than from specific reactions with one or more components of the succinate oxidase system. It seems most unlikely that these non-specific inhibitory effects of salicylate are related to *in vivo* actions of the drug since they were only observed at concentrations far greater than those attained either during medication or even after ingestion of lethal amounts of salicylate.

This general conclusion may not apply to the inhibitory effect of salicylate on the NADH–cytochrome c reductase system because the inhibition was reversed by NADH. The degree of inhibition produced by salicylate will depend on the concentration of NADH available to the enzyme system *in vivo* rather than on the

salicylate concentration reached and could be much more pronounced than with the succinate oxidase system. Thus relatively low concentrations of the drugs could interfere with the oxidation of NADH but not with the oxidation of succinate by tissues *in vivo*. It has been shown that both salicylate and resorcylate inhibit malate and isocitrate dehydrogenases *in vitro* by a mechanism involving reversible competition with NAD and also that they delay the metabolism of radioactive malate and citrate by animal tissue preparations.[3] The present results suggest that both drugs also interfere with the oxidation of NADH by the electron transport chain. Thus enzyme reactions requiring the participation of either NAD or NADH may be generally affected by salicylate and resorcylate in the tissues.

Gentisate differed from the other two drugs in causing an inhibition of the NADH–cytochrome c reductase system which was not reversed by NADH. This result agrees with the findings in the succinate oxidase system in that low concentrations of gentisate inhibit a component of the electron transport chain situated between the dehydrogenase flavoprotein and cytochrome c.

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