THE INHIBITION OF MALIC DEHYDROGENASE BY SALICYLATE AND RELATED COMPOUNDS

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Salicylate and γ-resorcylate affected the distribution of radioactivity incorporated from labelled succinate into the soluble intermediates of rat liver mitochondria in a manner consistent with an inhibitory action of the drugs on malic dehydrogenase. The addition of nicotinamide adenosine dinucleotide to the mitochondrial preparations reversed the effects of salicylate and γ-resorcylate. A general structural requirement for inhibitory activity against malic dehydrogenase *in vitro* in salicylate congeners appears to be a phenolic hydroxyl group in the *ortho* position to a carboxyl group except that 2-hydroxyphenylacetate also inhibited the enzyme.

Salicylate and γ-resorcylate have been reported to inhibit pig heart malic dehydrogenase activity *in vitro*; the mechanism of the inhibition involving competition with the coenzyme, nicotinamide adenosine dinucleotide (NAD) (Bryant, Smith and Hines, 1963). The present paper is concerned with the reversal by NAD of the changes caused by the drugs in the distribution of radioactivity incorporated from [1,4-^{14}C] succinate into the soluble intermediates of rat liver mitochondria. In addition a study has been made of the relation between chemical structure and the inhibitory action against malic dehydrogenase activity in congeners of salicylate.

Experimental

Materials

[1,4-^{14}C] succinate was obtained from the Radiochemical Centre, Amersham, Bucks, and pig heart malic dehydrogenase and NAD from C. F. Boehringer and Soehne. The salicylate congeners were obtained commercially and recrystallised until their melting-points remained constant. They were dissolved in glycine buffer at pH 9·6 (Gomori, 1955) to give solutions, which after admixture with the other constituents of the reaction mixtures used for the measurement of malic dehydrogenase activity, produced final concentrations of the drugs ranging from 5 to 30 mM.

Radioactive Experiments

Rat liver mitochondria, separated from 12 g. wet weight of liver by the method of Schneider and Hogeboom (1950) were finally suspended in 2 ml. of 0·25M sucrose. 50 μl. samples of the suspension were mixed with 25 μl. of a solution containing 0·5 μc of [1,4-^{14}C] succinate, 0·03 mM cytochrome C, 0·1 mM adenosine triphosphate, 1·0 mM adenosine diphosphate, 2·0 mM $MgSO_4.7H_2O$ and 10·0 mM KCl dissolved in 0·1 M potassium phosphate buffer, pH 7·4 (Gomori, 1955) and incubated for 30 min. at 37°. Salicylate and γ-resorcylate, when present, were added in the phosphate buffer to give final concentrations of 5 mM and NAD was also added in some experiments to give a final concentration of 3·25 mM. At the end of the incubation period the mitochondria were killed by the

addition of 300 μl. of boiling ethanol and the radioactive substances in the ethanolic extract were separated by two-dimensional paper chromatography, visualised by radioautography, and the ^{14}C measured by the techniques described by Smith and Moses (1960).

TABLE I

EFFECTS OF 5 mM SALICYLATE AND 5 mM γ-RESORCYLATE ON THE METABOLISM OF [1,4-^{14}C] SUCCINATE BY RAT LIVER MITOCHONDRIA IN THE PRESENCE OR THE ABSENCE OF 3·25 mM NAD

Results expressed as 10^{-3} × counts/min.

Soluble intermediate	Control	Control + NAD	Salicylate	Salicylate + NAD	Resorcylate	Resorcylate + NAD
Succinate	0·9	1·8	1·0	1·5	1·4	1·1
Fumarate	9·7	5·4	12·0	5·0	14·4	6·2
Malate	20·6	15·0	36·4	16·4	43·1	13·5
Citrate	0·4	0·2	0·9	0·3	0·6	0·2
Aspartate	2·1	2·1	2·0	2·3	1·3	3·3
Glutamate	2·1	1·7	0·8	1·3	0·5	1·0
Alanine	0·5	0·6	0·2	0·7	0·2	0·4

TABLE II

INHIBITION OF PIG HEART MALIC DEHYDROGENASE ACTIVITY BY SALICYLATE CONGENERS

The results, expressed as mean percentage inhibitions, have been analysed by the *t*-test and values of P are included. The minimum acceptable level of significance has been taken as P = 0·02.

Congener	No. of observations	Conc. (mM)	Per cent inhibition after 30 sec.	P
Salicylate	6	10	34·6	0·001
γ-Resorcylate	6	5	54·3	0·001
Benzoate	6	30	5·0	0·2
Phthalate	5	10	3·7	0·1
2-Methoxybenzoate	4	10	2·5	0·4
2-Hydroxyphenylacetate	6	10	29·6	0·001
3-Hydroxybenzoate	4	10	2·5	0·7
4-Hydroxybenzoate	6	10	0	—
Phenol	6	30	5·0	0·1
trans-Hexahydrosalicylate	4	10	4·1	0·3

Measurement of Malic Dehydrogenase Activity

The commercial enzyme was dialysed against phosphate buffer, pH 7·4, during which procedure it was diluted twenty times, and the dialysed enzyme was then diluted 1 to 100 with glycine buffer, pH 9·6 before use. The reaction mixtures contained 1 ml. of 30 mM sodium malate, 1 ml. of 0·75 mM NAD, 0·01 ml. of the enzyme preparation and 1 ml. of either glycine buffer or a solution of the salicylate congener in glycine buffer. The enzyme activity was estimated by measuring the change in optical density at 340 mμ in a Hilger Uvispek spectrophotometer at 15 sec. intervals over a period of 2 min. (Burton and Wilson, 1953).

RESULTS

The results given in Table I show the amounts of radioactivity from the labelled succinate incorporated into the soluble intermediates of rat liver mitochondria. In the control experiments radiocarbon was found in fumarate, malate, citrate, aspartate, glutamate and alanine and the

addition of NAD decreased the incorporation of the isotope into these intermediates and presumably increased the conversion of succinate carbon to CO_2. The most prominent action of salicylate and γ-resorcylate was to increase the accumulation of ^{14}C into fumarate and malate but the addition of NAD reversed this effect.

Table II gives the results of the effects of the salicylate congeners on malic dehydrogenase activity *in vitro*. Salicylate, γ-resorcylate and 2-hydroxyphenylacetate significantly inhibited the enzyme activity but the other substances had no effect.

Discussion

The present results confirm the work of Huggins, Bryant and Smith (1961) and Bryant, Smith and Hines (1963) in that salicylate and γ-resorcylate increase the incorporation of ^{14}C into fumarate and malate of rat liver mitochondria incubated with labelled succinate. It has been reported that both drugs inhibit malic dehydrogenase activity *in vitro* and that the mechanism of inhibition involves competition with the coenzyme, NAD (Bryant, Smith and Hines, 1963). The results in Table I show that the presence of a large excess (3·25 mM) of NAD in the mitochondrial experiments reverses the most prominent effect of salicylate and γ-resorcylate, that is the increased formation of radio-active malate. Thus the addition of an adequate amount of the coenzyme appears to counteract the inhibitory action of the drugs on the mitochondrial malic dehydrogenase and prevent the increased accumulation of the labelled malate.

Only salicylate, γ-resorcylate and 2-hydroxyphenylacetate significantly inhibited malic dehydrogenase activity *in vitro*. If salicylate represents the parent molecule then absence of the hydroxyl group (benzoate), alteration of its position relative to the carboxyl group (3- and 4-hydroxybenzoates), substitution of it by a carboxyl group (phthalate) or its methylation (2-methoxybenzoate), all caused a loss of activity. The absence of the carboxyl group (phenol) and hydrogenation of the benzene ring (hexahydrosalicylate) also produced inactive compounds. A general structural requirement for inhibitory activity against the dehydrogenase in congeners of salicylate therefore appeared to be a phenolic hydroxyl group in the *ortho* position to a carboxyl group except that the introduction of a methylene group between the benzene ring and the carboxyl group (2-hydroxylphenylacetate) did not remove the activity.

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