INACTIVATION OF METALLOENZYMES BY SALICYLATE

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1. Introduction

During experiments designed to test the stability of enzymes to a number of reagents [1], it was shown that triosephosphate dehydrogenase and glutamic dehydrogenase became unstable in the presence of salicylate at a wide variety of concentrations and conditions, including therapeutic, and at physiological pH and temperatures [2]. We made the chance observation that triosephosphate and glutamic dehydrogenases were readily inactivated in control tubes when imidazole served as buffer. Although inhibition of alcohol dehydrogenase by imidazole has previously been noted [3], we are unaware of any study reporting inactivation of enzymes by this reagent. Indeed, imidazole had been used extensively as an innocuous buffer. Since imidazole is an efficient metal binding agent, we checked the possibility that there was a correlation between enzyme stability to salicylate, a good metal chelator [4], and metal requirements [5]. A comparison of stability to salicylate and imidazole of a number of enzymes is presented here; the data indicate a close relationship between stability to these reagents and metal requirements.

2. Materials and methods

Sodium salicylate was obtained from Fisher Scientific Company; imidazole, pig heart malic dehydrogenase, bovine liver glutamic dehydrogenase, urease (Type V), ribonuclease, phosphoenol pyruvate carboxylase and Gum Carrageenin were obtained from Sigma. Methylsalicylic acid, 3-*tert*-butyl-6-methylsalicylic acid, *p*-hydroxybenzoic acid and meta-hydroxybenzoic acid were obtained from Aldrich Chemical Co. Enolase, 3-phosphoglycerate mutase (muscle and yeast) and acyl phosphatase were prepared as previously described [6–8]. All other reagents were commercial reagents.

Female Sprague-Dawley rats, weighing 150–200 g were used for these studies. Standard assays were employed in measuring triosephosphate dehydrogenase [9], glutamic hydrogenase [10], alcohol dehydrogenase [11], malic dehydrogenase [12], phosphoenol pyruvate carboxylase [13], acyl phosphatase [8], urease [14], ribonuclease [15], enolase and mutase [7] activities. Proteins were determined by a modification of the biuret method [16]. Rats were fed standard Purina Rat Chow to which imidazole or sodium salicylate were added to give 1% concentrations.

To produce paw edema in the rat, 0.1 ml of a 0.5% solution of Gum Carrageenin was injected subcutaneously in the dorsum of a hind foot of lightly anesthetized (ether) animals which had been on one of the previously described diets for 6–8 days. The degree of swelling and redness was compared 30 min after injection. These studies were conducted in collaboration with Dr.D.Scarpelli.

The effects of imidazole and salicylate upon collagen-induced platelet aggregation [17] were carried out by Dr.J.Davis

3. Results and discussion

As illustrated in table 1, some enzymes are stable while others are not in the presence of salicylate or imidazole. Moreover, as shown, the effect of salicylate on stability parallels that of imidazole. In an effort to clarify these effects, we compared metal

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Table 1
Effect of imidazole and salicylate on stability of various enzymes.

Enzyme tested	% Remaining activity after 60 min in the presences of		Metal requirement
	Imiadazole	Salicylate	
Malic dehydrogenase	22	0	+
Enolase	1	0	+
Phosphoenol pyruvate carboxylase	0	0	+
Alcohol dehydrogenase	21	4	+
Triosephosphate dehydrogenase	27	3	+
Glutamic dehydrogenase	29	9	+
Muscle P-glycerate mutase	100	100	–
Yeast P-glycerate mutase	100	100	–
Ribonuclease	100	100	–
Acyl phosphatase	100	100	–
Urease	100	100	–

The incubation mixtures contained in 1 ml 20 μmoles of tris-Cl buffer, pH 7.0, when testing for stability of triosephosphate dehydrogenase; 200 μmoles of KPO_4 buffer, pH 7.5, when testing for stability of glutamic dehydrogenase; 200 μmoles of KPO_4 buffer, pH 7.0, when testing for stability of urease; and 200 μmoles of tris-Cl buffer, pH 7.0, when testing for stability of all other enzymes. The following amounts of enzyme were added: 1.0 mg of rabbit muscle triosephosphate dehydrogenase, 1.0 mg of bovine liver glutamic dehydrogenase, 0.1 mg of horse liver alcohol dehydrogenase, 2.4 mg of phosphoenol pyruvate carboxylase, 0.1 mg of heart acyl phosphatase, 1.0 mg of urease, 0.2 mg of ribonuclease, 0.48 μU. of pig heart malic dehydrogenase, 100 units of enolase, 100 units [6] of chicken or yeast P-glycerate mutase. When used, 400 μmoles of either imidazole or salicylate were added. Incubation was at 38°. At zero time and at 60 min, 5–10 μl aliquots were removed and assayed by the procedures referred to (see Methods). The controls remained entirely stable during the incubation. The activity at zero time was unchanged by the imidazole or salicylate used.

requirements, SH and subunit composition for the enzymes tested. These was no correlation between stability and SH composition or subunits but, as shown in table 1, there seems to be an excellent correlation between instability induced by both imidazole and salicylate and metal requirement [5]. For compactness of presentation, only data for a high concentration of salicylate and imidazole are shown. However, both imidazole and salicylate are effective also at lower concentrations as illustrated with two dehydrogenases in fig. 1.

It is of interest that *p*- and *m*-hydrobenzoic acid do not appreciably inactivate triosephosphate or glutamic dehydrogenases. This is illustrated in table 2 and 3.

In view of the above results, we tested the possibility that there was correlation between imidazole and salicylate effects in other biological systems. Rats were fed imidazole or salicylate in their diets for 5 days. The inflammatory response produced by 0.5% Gum Carrageenin was reduced in the groups of rats fed imidazole and paralleled the response obtained

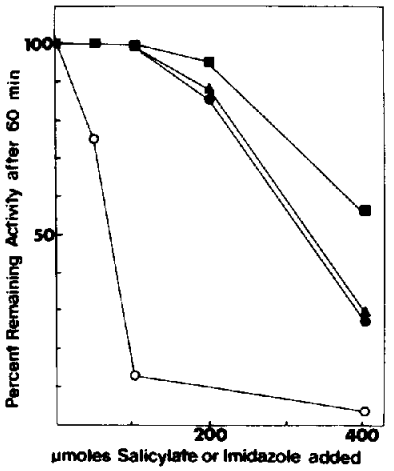

Fig. 1. The effect of imidazole and salicylate concentrations on enzyme stability. ■—■, glutamic dehydrogenase plus salicylate; ○—○, triosephosphate plus salicylate; ▲—▲, glutamic dehydrogenase plus imidazole; ●—●, triosephosphate plus imidazole. Details are given in legend to table 1.

Table 2
The effect of salicylate and related compounds on stability of glutamic dehydrogenase.

Compound	μmoles added	% remaining activity at (hr)		
		2	4	24
Sodium salicylate	10	100	100	80
Acetylsalicylate	10	100	100	80
5-Methylsalicylate	10	90	90	80
3-*tert*-Butyl-6-methylsalicylate	10	15	0	-
Sodium salicylate	20	100	100	70
Acetylsalicylate	20	100	100	70
5-Methylsalicylate	20	80	80	60
3-*tert*-Butyl-6-methylsalicylate	20	10	0	-
Sodium salicylate	50	90	80	50
Acetylsalicylate	50	90	80	50
5-Methylsalicylate	50	60	50	25
3-*tert*-Butyl-6-methylsalicylate	50	10	0	-
p-Hydroxybenzoate	50	90	90	90
m-Hydroxybenzoate	50	100	100	100
Benzoate	50	90	90	90

The conditions were as given in legend to table 1 except that the above compounds were added as indicated and the incubations were at 40°.

Table 3
The effect of salicylate and related compounds on stability of triosephosphate dehydrogenase.

	% Activity remaining at 1 hr μmoles added				
	10	20	30	40	50
Sodium salicylate	78	63	27	25	23
Acetylsalicylate	80	66	38	34	22
5-Methylsalicylate	63	48	25	24	23
3-*tert*-Butyl-6-methylsalicylate	26	23	23	20	15
p-Hydroxybenzoate	92	81	77	68	-
m-Hydroxybenzoate	97	85	84	73	-
Benzoate	100	100	90	81	-

Experimetal conditions are as given in legend to tables 1 and 2.

with animals fed salicylate. The salicylate and imidazole treated animals showed minimal signs of redness or swelling in contrast to control animals. Moreover, imidazole at 1 and 5 mM concentrations in 0.9% NaCl produced 51 and 58% inhibition respectively of collagen induced platelet aggregation with 0.9% NaCl as control. Sodium salicylate at the same concentrations produced 11 and 47% inhibition respectively.

The possibility that salicylates act therapeutically by forming chelates was considered many years ago [14]. Indeed, salicylate forms stable chelates with transition elements such as cobalt, iron and copper with binding constants of 10^5 to 10^9. The meta and para isomers of hydroxy-benzoic acid, which do not form chelates, are ineffective both as antiinflammatory and as antipyretic agents. As shown here the same appears to apply to *in vitro* stability to salicylate. According to Schubert and Lindenbaum [18], calcium and magnesium are not involved in salicylate action because they cannot displace the proton of the hydroxyl group of salicylate and because the binding constants are low; they suggested the participation of copper in salicylate action. It should be noted that predictions from binding constants [19] cannot be related to chelation *in vivo* or *in vitro* [20]. Whether the effects found with salicylate and imidazole are common or characteristic of all metalloproteins necessitates additional studies. However, in all cases tested, if one adds metals to the enzyme preparation the instability induced by salicylate and imidazole is prevented. In this context the investigation to find out if a number of metals, particularly zinc, as well as

other heavy metals both in animals and in patients which had received large amounts of salicylate should be of much interest. The effects upon several enzymes of feeding imidazole or salicylate to rats and rabbits are under study; it is too early to assess the observed changes and particularly whether or not we are dealing with effects on protein synthesis, degradation or both. Experiments are underway to correlate protein turnover and enzyme inactivation. With the enzymes tested thus far imidazole is in all cases more effective than salicylate which may reflect non-specific binding phenomena rather than more selective binding due to chelation. Possibly we may be simply dealing with subunit association since subunits may be held together by heavy metals.

The present findings are both of practical and theoretical interest, and may lead to a rational explanation of the phenomonological aspects of salicylate action. Such information may provide the basis for a better understanding and treatment of diseases such as rheumatic fever or rheumatoid arthritis. Possibly reagents such as imidazole or derivatives thereof which may have less untoward effects than salicylates may prove to be useful in medicine.

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