THE ACTION OF SALICYLATES AND RELATED COMPOUNDS ON THE SULPHATE EXCHANGE OF CHONDROITIN SULPHURIC ACID

BY HARRY BOSTRÖM and BENGT MÅNSSON

From the Chemistry Department II, Karolinska Institutet, Stockholm, Sweden

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THE beneficial effect on certain rheumatic diseases of various naturally occurring salicylates appears to have been known for several hundred years. Salicylic acid, whose antirheumatic action was demonstrated independently by Stricker[1] and by MacLagan[2] in 1876, still retains its position as one of the most important remedies in the treatment of rheumatic fever. In addition to salicylic acid and sodium salicylate, a number of other salicylates and related compounds have been tested as antirheumatics[3]. However, only few of these compounds, e.g., salicylamide[4], gentisic acid[5] and γ-resorcylic acid[6], have been found to have an effect comparable to that of salicylic acid. An interesting fact is that the two isomers of salicylic acid, *m*-hydroxybenzoic acid and *p*-hydroxybenzoic acid, lack any therapeutic effect in rheumatic fever.

The use of salicylates in the treatment of rheumatic fever is based on empirical findings. Although during past decades much experimental and clinical evidence has been accumulated, and many theories have been discussed, fundamental problems regarding the biological mechanism of action of the salicylates in rheumatic fever remain unsolved.

During recent years, the discussions in the literature have been mainly centred on two theories. According to one of them, the antirheumatic action is correlated to a hyaluronidase-inhibiting effect of salicylates. The second modern theory is that the action of salicylates in rheumatic fever is to be ascribed to stimulation of the anterior pituitary and adrenal cortex, with the resulting production of adrenal corticosteroids, which are the active therapeutic agents. Although several clinical and experimental findings seem to support these theories, both are open to much criticism, as was pointed out recently in a survey by Smith[7].

Since the pathological manifestations of rheumatic disease occur in some mesenchymal tissues, e.g., connective tissue, cartilage and tendons, it appears probable that drugs useful as antirheumatics would influence the metabolism of certain of their chemical constituents. Among the important compounds present in the aforementioned tissues are collagen, elastin and mucopolysaccharides of different kinds, such as hyaluronic acid, chondroitin sulphuric acid and heparin. Our knowledge of the metabolism of these compounds is, however, far from complete. With regard to the metabolic activity of collagen, it was demonstrated recently by Neuberger, Perrone and Slack[8] that, in tendons of adult rats, this activity is extremely low. This indicates that collagen once deposited in the intracellular spaces probably becomes metabolically inert.

In the case of mucopolysaccharides, most studies on the metabolism of these compounds have been confined to problems concerning the enzymatic exchange of the sulphate groups in sulpho-mucopolysaccharides, and have been made with the use of ^{35}S-labelled sulphate as the tracer substance[9,10,11]. On the basis of these studies recently reviewed[11,12], the following statements can be made. (A) The sulphate groups of sulpho-mucopolysaccharides are in a dynamic state, and the biological half-life period of the sulphate groups of chondroitin sulphuric acid of the skin and costal cartilage of adult rats amounts to about 9 and 16 days, respectively. (B) The sulphate exchange of chondroitin sulphuric acid can easily be demonstrated in *in vitro* experiments, e.g., in slices of surviving cartilage from calves, using methods described previously[13]. The reaction studied is of an enzymatic nature; it can be inhibited by various enzyme inhibitors, of which SH reagents are the most potent, and can be stimulated by the presence of a hitherto unidentified factor in the liver[14]. (C) *In vivo* and *in vitro* experiments have shown that cortisone decreases the sulphate exchange of chondroitin sulphuric acid[15,16].

As far as the similarity in the response to cortisone and to certain salicylates in rheumatic disease is concerned, a question of interest is the influence of salicylates and related compounds on the sulphate exchange of chondroitin sulphuric acid. The object of the present investigation was a study of this problem. The aforementioned *in vitro* technique was used, and 10 different compounds were tested.

EXPERIMENTAL

Reagents

1. Krebs-Ringer-bicarbonate solution.*
2. ^{35}S-labelled Krebs-Ringer-bicarbonate solution. To 400 ml. of the ordinary Krebs-Ringer-bicarbonate solution (reagent 1) were added 10 mC. of carrier-free ^{35}S-labelled sodium sulphate† in approximately 0·5 ml. of distilled water. The solution was poured into 50-ml. flasks, frozen, and stored as solid. The reagent was used over a period of 2 to 3 months, the same solution being used in all the experiments described in this paper. Before use, the content of one of the flasks was thawed and used in one or two experiments. The radioactivity of this isotope solution at the beginning of the experiments was of such an order of magnitude that, if 1 ml. of the solution was added to 25 ml. of reagent 1, the radioactivity of the final solution amounted to 2×10^6 c.p.m. per sq. cm. (measured at $BaSO_4$ at infinite thickness).
3. Monoiodoacetic acid, a 2 per cent. solution in distilled water.
4. The salicylates and related compounds tested were of the highest commercial purity available; most of them were recrystallised several times.

* Prepared according to the directions given in Umbreit, Burris and Stauffer: "Manometric Techniques and Tissue Metabolism" (p. 119), Burgess Publishing Co., Minneapolis 15, Minn. 1951.

† Obtained from A.E.R.E., Harwell, England.

METHODS

The compounds to be tested were dissolved in 25 ml. of Krebs-Ringer-bicarbonate solution (reagent 1) to a concentration of 4 mM/l. The *p*H of the solutions was checked and, if necessary, adjusted to *p*H 7·4. The solutions were then poured into 100-ml. Erlenmeyer flasks, to which 5 g. of 0·3 mm. thick slices of costal cartilage from newly killed suckling calves were added[13]. The flasks were rocked in a water bath at 37° C. for 2 hours, and aerated with a gas mixture (93·5 per cent. oxygen plus 6·5 per cent. carbon dioxide). After this time, 1 ml. of the ^{35}S-labelled Krebs-Ringer-bicarbonate solution (reagent 2) was added to each flask. The flasks were then rocked again to and fro in the water bath, and aerated with the oxygen-carbon dioxide mixture for 2 hours. After incubation, the reaction was stopped by adding 1 ml. of 2 per cent. monoiodoacetic acid (reagent 3) to the samples. Chondroitin sulphuric

TABLE I

INFLUENCE OF DERIVATIVES OF SALICYLIC AND BENZOIC ACIDS ON THE ^{35}S UPTAKE OF CHONDROITIN SULPHURIC ACID

Substance	^{35}S-uptake in chondroitin sulphuric acid Percentage of controls*			Percentage inhibition Mean value
	1	2	3	
Controls	100	100	100	—
Sodium salicylate	69	73	74	28
Acetylsalicylic acid	86	81	86	16
Sodium *p*-aminosalicylate	96	100	101	1
Salicylamide	93	90	92	8
Sodium gentisate	102	92	99	2
2:4-Dihydrobenzoic acid	92	98	102	3
o-Aminobenzoic acid	92	90	93	8

* Slices of cartilage incubated without the addition of salicylates.

acid was then prepared from all the samples according to methods described previously[17]. The radioactivity of the different samples of chondroitin sulphuric acid was measured in a Geiger-Müller counter. The values are given as counts/min./sq. cm. of the sodium salt of chondroitin sulphuric acid at infinite thickness.

In each experiment, 18 or 20 flasks were incubated simultaneously. In one series of experiments, the ^{35}S incorporation in chondroitin sulphuric acid in 4 controls (slices incubated without the addition of salicylates) was compared with that in 4 quadruple determinations on samples incubated in the presence of sodium salts of benzoic acid: *o*-, *m*-, and *p*-hydroxybenzoic acid, respectively. In another series, the ^{35}S uptake in 4 controls was compared with that in double determinations on samples containing one of the following: sodium salicylate, sodium gentisate, 2:4-dihydroxybenzoic acid, *o*-aminobenzoic acid, acetylsalicylic acid, salicylamide or the sodium salt of *p*-aminosalicylic acid. Both these types of experiments were repeated 3 times. The standard deviation of a double and a quadruple determination amounted to 3·6 and 2·5 per cent. respectively.

RESULTS

The results of the experiments are recorded in Figures 1 and 2 and Table I. As shown in Figure 1, the presence of 4 mM of sodium salicylate in the medium inhibited the *in vitro* ^{35}S incorporation in chondroitin sulphuric acid by about 26 per cent. On the other hand, no significant inhibition was caused by equimolar concentrations of the two isomers of this compound, the sodium salts of *m*-hydroxybenzoic acid and *p*-hydroxybenzoic acid. Slight inhibition, amounting to 16 per cent., was caused by sodium benzoate. Of the compounds tested in the experiments illustrated in Figure 2 and Table I, only sodium salicylate and acetylsalicylic acid produced a

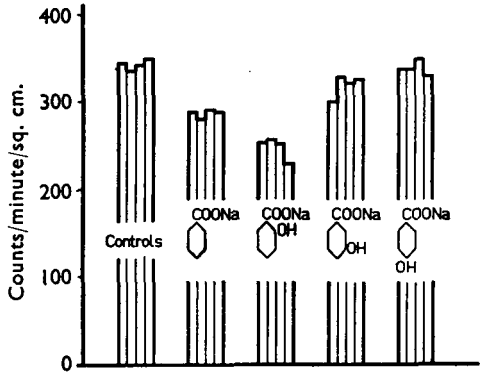

FIG. 1. Influence of the sodium salts of benzoic acid, salicylic acid, *m*- and *p*-hydroxybenzoic acid on the ^{35}S incorporation of chondroitin sulphuric acid as studied *in vitro* in slices of cartilage.

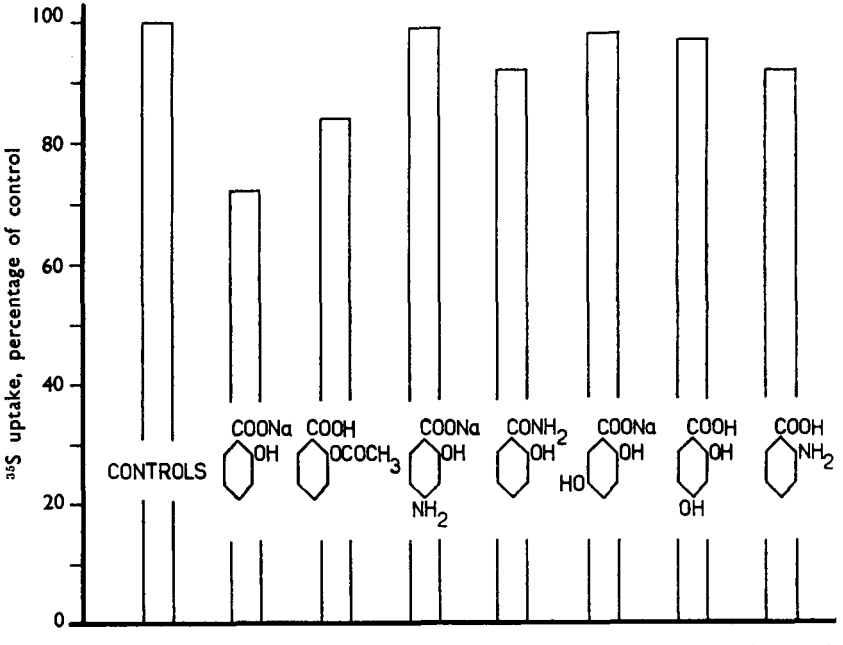

FIG. 2. Diagram showing the chemical structure of different compounds tested, and their effect on the ^{35}S uptake in cartilage. Mean values of all the experiments recorded in Table I.

significant inhibition (28 and 16 per cent., respectively). The other compounds, i.e., sodium gentisate, 2:4-dihydroxybenzoic acid, salicylamide, *o*-aminobenzoic acid and the sodium salt of *p*-aminosalicylic acid, had no significant influence on the ^{35}S uptake in chondroitin sulphuric acid.

Discussion

The mechanism of the therapeutic action of salicylates is not completely understood. Most modern workers seem, however, to agree that the effect of these drugs is to be ascribed to their action on mesenchymal tissues. On the other hand, opinions differ regarding whether or not salicylates exert this effect by stimulation of the anterior pituitary and adrenal cortex[7,18].

In the present study on surviving cartilage, the influence of hormonal systems can be disregarded. Consequently, the inhibition of the sulphate exchange in chondroitin sulphuric acid caused by some of the compounds tested seems to be due to a direct action on the cartilaginous tissue. With regard to the specificity of this inhibition, it is of some interest to note that the most commonly used antirheumatic of this type, sodium salicylate, caused the greatest degree of inhibition. The two isomers of this compound, the *m*-hydroxybenzoate and *p*-hydroxybenzoate, which are therapeutically inert[7], had, on the contrary, no influence on the reaction in question. Acetylsalicylic acid and sodium benzoate caused only slight inhibition of the sulphate exchange of chondroitin sulphuric acid. None of the other compounds tested had any such inhibitory effect. Of these compounds, *p*-aminosalicylic acid, *o*-aminobenzoic acid and 2:4-dihydrobenzoic acid are not useful as antirheumatics, whereas—according to recent reports[4,5]—gentisic acid and salicylamide seem to have an antirheumatic action.

The concentration in the suspending medium of the compounds tested in the present experiments amounted to 4 mM/l., corresponding to 55 mg./100 ml. (in the case of sodium salicylate). This concentration is only slightly higher than the plasma salicylate levels reported by Coburn[19] in cases of rheumatic fever treated with large doses of sodium salicylate.

The nature of the inhibition by sodium salicylate of the reaction studied is not fully elucidated. Interesting parallels to this observation are, however, found in recent reports. Thus, it has been shown in *in vitro* and *in vivo* experiments that cortisone inhibits the sulphate exchange of chondroitin sulphuric acid[15,16] and that cortisone and sodium salicylate administered to rats cause a significant decrease in the hexosamine content of the skin[20]. Finally, cortisone and sodium salicylate have been found to inhibit the production of experimental arteritis in rabbits[21]. All these observations seem to substantiate the view that both cortisone and sodium salicylate decrease the synthesis and metabolic activity of mucopolysaccharides in the mesenchymal tissues.

Summary

1. The action of different salicylates and related compounds on the sulphate exchange *in vitro* of chondroitin sulphuric acid in slices of cartilage has been studied.

2. Sodium salicylate is found to exert a highly inhibitory effect on this reaction and acetylsalicylic acid and benzoic acid a slight effect.

3. No inhibition is found to result from *m*- and *p*-hydroxybenzoic acid, gentisic acid, salicylamide, the sodium salt of *p*-aminosalicylic acid, or *o*-aminobenzoic acid.

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