Effect of Metal-Complexing Agents on Mucopolysaccharide Sulfate Biosynthesis

By WILLIAM O. FOYE, MARIA C. M. SOLIS, J. W. SCHERMERHORN, and EDWIN L. PRIEN

A series of substituted salicylates and other structurally unrelated compounds having the common property of metal-ion complexation have been examined for the ability to inhibit the incorporation of sulfate into mucopolysaccharide. Several very potent inhibitors have been found among the mercaptoamines. It may be stated that the course of mucopolysaccharide sulfation is definitely affected by metal-binding agents, generally by inhibition. A close correlation between per cent inhibition of sulfation and metal-binding strength was observed, which establishes that the mode of inhibition is one of metal binding of the metalloenzymes involved in mucopolysaccharide sulfation.

SALICYLATES have been shown to inhibit the incorporation of ^{35}S into cartilage mucopolysaccharide (1). Whitehouse and Bostrom (2) have postulated that salicylate exerts its inhibitory effect on this reaction either by acting as an alternative sulfate acceptor, being itself sulfated, or by complexation of the metalloenzymes involved in mucopolysaccharide sulfation (2, 3). The results reported here permit a more definite conclusion to be made.

A series of substituted salicylates and other structurally unrelated compounds having the common property of metal-ion complexation were tested for the ability to inhibit the incorporation of ^{35}S into veal costal cartilage, according to the procedures of Rubin and Howard (4) and Whitehouse and Bostrom (2). It may be stated that metal complexation agents definitely affect the sulfation, generally by inhibition. A correlation between metal-binding strength and degree of inhibition is apparent, although agents with comparatively high or low metal-binding constants were found to stimulate the incorporation of sulfate.

METHODS

Incubation Procedure.—Incubations were carried out at 37° in air with continuous slow shaking. Costal cartilage from a newly killed calf was sliced to 0.5 mm. thickness and in each determination five slices were preincubated in 10 ml. of Krebs-Ringer solution with the inhibitory agent (5×10^{-3} *M*). After 15 min., sodium sulfate ^{35}S (5×10^{5} counts/min.) was added, and incubation was continued for 3 hr. The medium was then decanted, and the slices were washed three times with chilled (4°) Krebs-Ringer solution, blotted, and stored in the moist state at 4° until taken for counting.

Measurement of Incorporated Radioactivity.—*Approximate (Direct) Assay.*—The intact, moist, circular cartilage slices (dry weight approximately 40 mg.) were placed in tared planchets and dehydrated by standing overnight in the refrigerator and drying for 10 min. at 100°. The planchets were weighed, and the radioactivity of the slices was measured directly with an end-window flow counter (Picker Compact Flow Detector). Results are recorded in Table I.

Assay of Digested Slices.—Papain (2.66 mg. for 40 mg. of tissue) was activated by admixture to a paste with a few milliliters of 0.1 *M* phosphate buffer (pH 6.8) containing 1.22 mg./ml. of cysteine hydrochloride (0.005 *M*). The paste was diluted to the desired volume with phosphate buffer and warmed at 55° for 30 min. The dried cartilage slices in phosphate buffer (1 ml.) were denatured in boiling water for 15 min. and treated with 1 ml. of activated papain suspension. They were digested overnight at 55° and retreated with papain if still undigested. A small residue was removed by centrifugation. A 1-ml. aliquot of the clear liquid was placed in a planchet, dried, and the incorporated ^{35}S was measured. (See Table I.)

The total ^{35}S (free and polysaccharide-bound) taken up by the tissues was thus measured, but it has been shown that 92% of the total ^{35}S is incorporated into mucopolysaccharide after 30 min. incubation (2). Each series of incubations included at least one contamination control, in which the

TABLE I.—INHIBITORY EFFECTS OF METAL-BINDING AGENTS ON ^{35}S INCORPORATION IN MUCOPOLYSACCHARIDE

Agent, 5×10^{-3} *M*	% Inhibition, Incubated Slices	% Inhibition, Digested Slices
Salicylic acid	40.20	43.86
o-Thymotic acid	62.46	48.30
3,6-Dimethylsalicylic acid	35.80	21.97
5-Nitrosalicylic acid	21.40	14.90
4-Aminosalicylic acid	−18.70[a]	−30.80[a]
Salicylamide	−36.60[a]	−47.40[a]
2-Mercaptoethylamine	90.40	93.40
2-Mercaptoethylguanidine	62.90	71.90
2-Mercaptoethylguanidine trithiocarbonate	93.31	95.60
Pyridine-2-thiol-1-oxide Zn[b]	86.24	92.51
Na Diethyldithiocarbamate	70.03	72.84
8-Hydroxyquinoline	76.63	79.95
5-Nitrofurantoin	65.10	66.62
2-Mercaptobenzothiazole[b]	54.85	51.67
L-Cysteine·HCl	46.64	33.03
Ethylenediaminetetraacetic acid	33.10	22.80
Riboflavin[b]	6.10	−15.70[a]
Glycine	− 3.30[a]	0.46
Methionine	−18.30[a]	−23.20[a]

[a] Stimulatory effect. [b] Incompletely dissolved.

Received April 1, 1965, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.
Accepted for publication June 2, 1965.
Presented to the Scientific Section, A.PH.A., Detroit meeting, March 1965.
Abstracted in part from a thesis submitted by Maria C. M. Solis in partial fulfillment of Master of Science degree requirements.
Some of this work was carried out in the Squibb Radioisotopes Laboratory, Massachusetts College of Pharmacy.
The authors acknowledge the financial support of the Foundation for Clinical Research.

TABLE II.—STABILITY CONSTANTS OF INHIBITORS WITH METAL CATIONS

Agent	Log K_1 (Water, 20–30°)[a]					
	Cu^{2+}	Zn^{2+}	Co^{2+}	Fe^{2+}	Mn^{2+}	Fe^{3+}
Salicylic acid	10.6	6.9	6.8	6.6	5.9	16.4, 29.3*
o-Thymotic acid[b, c]	9.7	...	...	...	...	15.8
3,6-Dimethylsalicylic acid[d]	9.1	...	...	...	...	14.8
5-Nitrosalicylic acid[b, c]	...	...	...	...	...	14.5
4-Aminosalicylic acid[b, c]	...	...	...	...	...	16.3, 30.5*
Salicylamide	...	...	...	...	...	10.0
2-Mercaptoethylamine	...	10.2	7.7	...	...	...
Pyridine-2-thiol-1-oxide[e]	...	...	5.5	...	...	...
Diethyldithiocarbamate[f]	11.9	...	...	...	...	...
8-Hydroxyquinoline	12.2	8.5	9.1	8.0	6.8	12.3
Cysteine	...	9.8	9.3	6.2	4.1	32.1*
Ethylenediaminetetraacetic acid[g]	18.8	16.2	16.2	14.3	13.6	25.1
Riboflavin	13.0*	5.6	3.9	7.1	3.4	...
Glycine	8.5	5.2	5.1	4.3	3.2	10.0
Methionine	14.7*	4.4	7.9*	6.7*	...	...

[a] 1:1 Complexes are reported; 2:1 complexes are indicated by an asterisk. Values are taken from Bjerrum, J., Schwarzenbach, G., and Sillen, L. G., "Stability Constants, Part I: Organic Ligands," The Chemical Society, London, England, 1957, unless otherwise noted. [b] Determined in aqueous ethanol. [c] Foye, W. O., and Turcotte, J. G., *J. Pharm. Sci.*, **51**, 329(1962). [d] Baum, M. D., M.S. thesis, Massachusetts College of Pharmacy, Boston, 1964. [e] Albert, A., Rees, C. W., and Tomlinson, A. J. H., *Rec. Trav. Chim.*, **75**, 819(1956). [f] Janssen, M. J., *ibid.*, **76**, 827(1957). [g] Values represent the over-all stability constants.

tissue slices were incubated with iodoacetate at 37°. The very low radioactivity of these slices showed that extraneous sulfate-^{35}S was being removed by the washing procedure.

RESULTS AND DISCUSSION

The substituted salicylic acids selected as inhibitors cover a wide range of metal-binding strength. (See Table II.) The alkyl-substituted acids, the 3,6-dimethyl and 3-isopropyl-6-methyl (*o*-thymotic acid) derivatives, have avidities for heavy metals comparable to that of salicylic acid itself and show similar degrees of inhibition of mucopolysaccharide sulfation. The 5-nitro derivative is both a weaker complexing agent and sulfation inhibitor. The 4-amino derivative has a higher metal-binding constant for Fe^{3+} ion, and salicylamide a much lower iron-binding constant than salicylic acid. Both of these compounds, however, stimulated the uptake of ^{35}S, suggesting that complexation of a metalloenzyme here aids the sulfation rather than hinders it.

Among the other metal-binding agents tested for inhibition of sulfation are found a variety of structures, including a mercaptoamine, mercaptoguanidine, dithiocarbamate, trithiocarbonate, oxine, EDTA, and α-amino acids. The highest per cent inhibitions of sulfation were given by 2-mercaptoethylguanidine trithiocarbonate, 2-mercaptoethylamine, and pyridine-2-thiol-1-oxide zinc complex. No metal-binding constant is known for the trithiocarbonate, since it loses carbon disulfide in the presence of metal ions in aqueous solution and forms a mercaptoethylguanidine–metal complex (5), but metal-binding constants greater than those for salicylic acid have been reported for 2-mercaptoethylamine (MEA). Only one constant has been recorded for pyridine-2-thiol-1-oxide, which is slightly lower than the corresponding constant for salicylic acid. More constants should be available for comparison before this compound can be declared an exception in this correlation of sulfation inhibition and metal-binding strength (*e.g.*, compare the constants of salicylic acid and cysteine). A constant of somewhat greater magnitude than that of salicylic acid with cupric ion is known for diethyldithiocarbamate, and the degree of inhibition appears at a somewhat higher level. An instability constant for 2-mercaptoethylguanidine and cupric ion has been reported (6) which places this compound slightly lower in complexing avidity than MEA, and the per cent of sulfation inhibition is correspondingly lower as well.

Oxine (8-hydroxyquinoline) shows somewhat higher metal-binding constants than salicylic acid, and also shows a higher degree of inhibition of sulfation. L-Cysteine, however, shows a higher binding constant for Zn^{2+} and Co^{2+} than does salicylic acid, a comparable value for Fe^{2+}, but a lower value for Mn^{2+}. The degrees of inhibition of sulfation are very close for these two compounds. EDTA and salicylic acid are also comparable in both abilities. Glycine, methionine, and riboflavin have significantly lower metal-binding constants than has salicylic acid, and they show stimulatory effects on sulfate uptake.

5-Nitrofurantoin shows a somewhat greater ability to inhibit sulfate uptake than does salicylic acid, but this compound is not known as a metal-binding agent. It has been shown to bind significant amounts of calcium in our laboratory, however (7). Metal-binding constants for 2-mercaptobenzothiazole have not been reported, but constants somewhat lower than those for a dithiocarbamate may be expected. This would place the compound at the same inhibitory level as salicylic acid, which result was found.

A statistical treatment of the per cent inhibition data, using the values obtained for iodoacetate in each run, showed a reproducibility between runs of 2.62–2.97. Using the Student *t* distribution treatment, no significant differences in the values for incubated and digested tissue slices were found, and it also appeared that the cartilage used was essentially the same for all runs.

Whitehouse (3) has studied the mucopolysaccharide sulfation reaction in an attempt to explain anti-inflammatory properties of drugs acting on connective tissues. Another biological implication of this reaction is also apparent.

The presence of mucopolysaccharide sulfates in renal calculi has led to their implication as centers of nucleation for stone growth (4, 8). It is possible that several of the agents that have been employed clinically for halting stone growth, such as the salicylates and 5-nitrofurantoin, may be effective because of their ability to inhibit the formation of mucopolysaccharide sulfates.

It does appear from the data here, however, that this reaction is inhibited because of the metal-binding properties of molecules for the metalloenzymes involved in mucopolysaccharide sulfation. The enzymes that are known to be involved in this reaction include ATP-sulfurylase, ADP-sulfurylase, APS-kinase, and sulfokinase. Magnesium has been identified as a cofactor, but other metal ions may be involved.

REFERENCES

(1) Bostrom, H., and Mansson, B., *J. Pharm. London*, **7**, 185(1955).
(2) Whitehouse, M. W., and Bostrom, H., *Biochem. Pharmacol.*, **7**, 135(1961).
(3) Whitehouse, M. W., *Nature*, **194**, 984(1962).
(4) Rubin, P. S., and Howard, J. E., "Conference on Metabolic Interrelations," Trans. 2, Reifenstein, E. S., ed., Josiah Macy, Jr., Foundation, New York, N. Y., 1950, pp. 155–166.
(5) Foye, W. O., and Mickles, J., *J. Pharm. Sci.*, **53**, 1030 (1964).
(6) Knoblock, E. C., and Purdy, W. C., *J. Electroanal. Chem.*, **2**, 493(1961).
(7) Unpublished data.
(8) Boyce, W. H., Garvey, F. K., and Norfleet, C. M., *J. Urol.*, **72**, 1019(1954).

Intermolecular Association of Stereoisomers as Examined by Infrared Spectra

By N. H. CHOULIS

Studies of acid–base association of asymmetric molecules in the solid state involving the determination of infrared spectra of diastereoisomeric salts are examined. Although infrared spectra measurements could not be used to indicate configuration of the components, definite differences in the spectra of salts formed from stereoisomers of like configuration from those of unlike configuration could be determined.

DIFFERENCES in the infrared spectra of various diastereoisomeric salts may be interpreted in terms of the difference in association of the components of these salts. Previously, Eliel and Kofron (1) found that differences in infrared spectra were significant enough to distinguish between an active and a racemic form of a substance.

Rosenberg and Shotte (2) examining the infrared spectra of quasi-racemic compounds found also differences in the infrared spectra of (+)-α,α-dimethylglutaric acid/(−)-α-methylglutaric acid and (+)-α,α-dimethylglutaric acid/(+)-α-methylglutaric acid.

Gronowitz (3) showed a difference in the infrared spectra of (+)-2-thienylsuccinic acid/(+)-3-thienylsuccinic acid and (−)-2-thienylsuccinic acid/(+)-3-thienylsuccinic acid. However, no attempt was made to examine the infrared spectra and the differences occurring between diastereoisomeric salts.

EXPERIMENTAL

Materials.—D-Amphetamine, L-amphetamine, D-α-phenethylamine, L-α-phenethylamine, D-mandelic acid, L-mandelic acid, D-tartaric acid, L-tartaric acid, and *meso*-tartaric acid were used.

The signs D- and L- refer to the absolute configuration of the compounds used.

Preparation of the Salts.—Equimolar quantities of the appropriate acid and base were dissolved in the appropriate solvent (*e.g.*, ethyl or methyl alcohol or ether, etc.) under reflux. The solution was cooled and placed in the refrigerator until crystals were separated. These crystals were filtered off, dried under vacuum, and melting points were taken; the salts were recrystallized to constant melting points.

Nujol mulls of the salts were prepared and placed between rock-salt plates, and the spectra were obtained.

Instrument.—The Unicam S.P. 200 was used.

RESULTS

The results showed that the salts formed from optically active acids and optically active bases of similar configuration had different spectra from those of the corresponding salts formed from acids and bases possessing opposite configuration (Tables I–III). Furthermore, as expected, the spectrum of a D-acid/D-base was superimposable to the spectrum of the corresponding L-acid/L-base diastereoisomeric salt. Also the D-acid/L-base and the L-acid/D-base salts had the same infrared spectra but dissimilar to the spectra of D-acid/D-base or L-acid/L-base diastereoisomeric salts.

The spectrum of a salt formed from a racemic or optically inactive acid with one stereoisomer of an optically active base was superimposable with that of the same racemic or inactive acid and the other stereoisomer of the base (Table IV).

Received March 15, 1965, from the School of Pharmacy, University of Kansas, Lawrence.
Accepted for publication May 18, 1965.
This investigation was carried out at the Chelsea School of Pharmacy, London, England, under a grant from the Greek Foundation Scholarships.