Biochemical Pharmacology, 1961, Vol. 7, pp. 135–150. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON THE ACTION OF SOME ANTI-INFLAMMATORY AGENTS IN INHIBITING THE BIOSYNTHESIS OF MUCOPOLYSACCHARIDE SULPHATES

M. W. WHITEHOUSE* and H. BOSTRÖM

Department of Metabolic Research, Wenner-Gren Institute, University of Stockholm
and the
Department of Medicine, Serafimerlasarettet, Karolinska Institutet, Stockholm, Sweden

(*Received* 2 *February* 1961)

Abstract—Salicylic acid and anti-inflammatory steroids inhibit the uptake of sulphate ions by cartilage and cornea *in vitro* and may further inhibit the utilization of the intracellular sulphate ions for the biosynthesis of sulphated mucopolysaccharides. These activities are ascribed to a common action of the drugs upon processes generating ATP within the tissues. The drug-sensitivity of connective tissues may increase with their age. The possible relationship between the biochemical action of these drugs and their therapeutic value as anti-inflammatory agents is discussed.

Some novel methods for the micro-estimation and quantitative isolation of polysaccharide sulphates using 6:9-diamino-2-ethoxy-acridine lactate (Rivanol) are described.

MUCOPOLYSACCHARIDE sulphates are characteristic components of the mesenchymal tissues in animals (connective tissue, tendon, cartilage etc.). Of especial importance are the chondroitin sulphates A and C and keratosulphate,† which are present in costal cartilage, the cornea and the nucleus pulposus.[1] Corneas contain a further polysaccharide, related in composition to the chondroitin sulphates but with a low content of ester sulphate (about 2 per cent), which has been designated "chondroitin".[2]

Certain drugs which are employed for the clinical management of rheumatic diseases, will inhibit the biosynthesis of mucopolysaccharide sulphates. These drugs include the salicylates, cortisone and many of its chemical analogues which are of value as anti-inflammatory agents.[3] We have sought to determine the precise action of these drugs upon the biosynthesis of cartilage and corneal polysaccharide sulphates.

Layton[4, 5] first noted a decrease in the incorporation of inorganic sulphate-^{35}S into various animal tissues *in vitro* and *in vivo*, following the administration of cortisone. He suggested that cortisone inhibited the biosynthesis of chondroitin sulphate in these tissues. This inhibitory action of cortisone has since been amply confirmed in numerous *in vivo* studies with the repeated observation of loss of metachromasia (associated with anionic polymers) and reduced incorporation of radiosulphate into connective tissues.[6] That cortisone acts directly upon connective tissue to inhibit the

* Permanent address: Department of Biochemistry, University of Oxford, Oxford, England.

† These polysaccharides have the composition: chondroitin sulphate A = polyglucuronido-N-acetylgalactosamine-4-sulphate; chondroitin sulphate C = polyglucuronido-N-acetylgalactosamine-6-sulphate; keratosulphate = polygalactosido-N-acetylglucosamine-6-sulphate.

biosynthesis of the mucopolysaccharide sulphates was demonstrated with cartilage slices *in vitro*.[7] Further experiments with cartilage-forming tissue cultures showed that not only cortisone but also a large number of related steroids with anti-inflammatory activity, inhibit chondrogenesis and the biosynthesis of chondroitin sulphate(s).[8] Analyses of the cartilage formed in tissue culture have suggested that it is the sulphation, and not the biosynthesis of the unsulphated polysaccharide, which is primarily affected by these steroids.[9, 10]

Salicylates have been shown to inhibit the incorporation of sulphate-^{35}S into cartilage polysaccharides *in vitro*.[11, 8]

We now present evidence that these steroids and the salicylates, though chemically unrelated, may have a common action in regulating the uptake and utilization of inorganic sulphate by cartilage and corneal slices.

EXPERIMENTAL METHODS

Materials for incubation

All incubations were conducted in freshly mixed modified Krebs–Ringer phosphate solution, pH 7·4. Magnesium chloride (0·11 M) was used in place of magnesium sulphate (0·15 M) to constitute the Krebs–Ringer solution. Preparation of steroid emulsions: Steroids were commercial products checked for authentic melting points. Aliquots of methanolic solutions of the steroids were transferred to test tubes containing a measured quantity of Tween 20 (polyoxyethylene sorbitan monolaurate, Atlas Powder Company, Wilmington, Delaware, U.S.A.) added as a solution in methanol. The methanol was removed from the mixture on warming in a water bath at 50 °C under a stream of air, leaving a solution of the steroid in droplets of Tween and a very small volume of methanol ($<$0·05 ml). Krebs–Ringer solution was added with vigorous shaking. Opalescent solutions were discarded. These emulsions usually contained two parts of Tween for each part of steroid. The efficiency of emulsification (and determination of the steroid in solution) was determined by measuring the optical density at 246 mμ after filtration, and comparing with the absorption of standard solutions of the steroids (Δ^4-3-ones) in ethanol.

Phenolic acids used were recrystallized samples (kindly donated by L. Reio), checked for homogeneity by paper chromatography and high-voltage paper electrophoresis. Solutions of the potassium salts (pH 7·4) were prepared by addition of potassium carbonate to suspensions of the acids in Krebs–Ringer solution. The precise concentration of the phenolic salt was determined spectrophotometrically at 246 mμ. Solutions of the dihydroxybenzoic acids were prepared afresh immediately before each incubation.

Circular cartilage slices (approx. 7 mm diameter, dry weight approx. 5 mg) were prepared as previously described[12, 13] from new-born calves and from cattle up to the age of 2 years, within 4 hr of slaughter. Circular "slices" of whole cornea (7 mm diameter, dry weight approx. 5·5 mg) were prepared from freshly excised calf and cattle corneas by "punching out" with a cork borer. The intact corneas and the individual slices were moistened continuously with Krebs–Ringer solution throughout this operation. Slices from a number of corneas were pooled in Krebs–Ringer solution, ten of these slices then being withdrawn at random for each incubation. Cartilage slices for any one series of experiments, were all prepared from one animal.

Incubations were conducted at 37 °C in air or oxygen with continuous slow shaking. Twenty cartilage slices or ten corneal slices were pre-incubated in a 50-ml flask with 9 ml of Krebs–Ringer solution containing steroids, phenols or other additions. The amount of Tween in this solution never exceeded 2 mg. After 15 min pre-incubation, sodium sulphate-^{35}S (5×10^5 counts/min) in 1 ml Krebs–Ringer solution was added to each flask. Incubation were continued for periods of up to 8 hr. When terminated, the medium was poured through a filter and the optical density at 246 mμ measured. Slices from each incubation were washed three times by decantation with chilled (4 °C) Krebs–Ringer solution, lightly blotted and stored in the moist state at 4 °C until taken for counting.

In each series of incubations, one flask contained slices, sulphate-^{35}S and sodium iodoacetate (5×10^{-3} M) and served as a control.

Measurement of incorporated radioactivity

(a) *First approximate (direct) assay.* Intact moist slices were plated in groups of four per plate on the special recessed plates, described for method II by Boström *et al.*[13] They were then dehydrated by standing in the refrigerator overnight and finally dried for 10 min at 100 °C. Corneal slices were grossly distorted unless dehydrated slowly in the refrigerator. The radioactivity of each plate with its dried slices, was measured directly with an end-window Geiger–Müller tube. The reproducibility between individual plates prepared with slices from any one incubation, was usually within the range 5–8 per cent.

(b) *Assay on digested slices.* Dried slices were digested with papain and the digests fractionated with Rivanol as described below. Aliquots (50 μl) were withdrawn from the whole digests, plated in quadruplicate on sand-blasted aluminium disks and counted to give a measure of the total incorporated ^{35}S (inorganic sulphate-^{35}S and polysaccharide sulphate-^{35}S). Aliquots (100-μl) from the soluble residue after precipitation of the polysaccharide sulphates, were likewise plated and counted to give a measure of the inorganic sulphate-^{35}S alone. Reproducibility between plates prepared from the one digest, was excellent ($\pm$3 per cent). Self-absorption was negligible though self-absorption curves were prepared.

Isolation of polysaccharide sulphates

Portions of cartilage and cornea (approximately 40 mg dry weight) in phosphate buffer (0·1 M, pH 6·8) were denatured by heating in a boiling water bath for 15 min to facilitate the subsequent digestion of collagen-like proteins (cf. Gardell[14]), then digested overnight with papain at 55 °C.[15] (The activated papain was prepared from one part of crude papain per fifteen parts dry weight of tissue, and filtered before use.) A small tissue residue resisted digestion (usually 5–10 per cent original dry weight) and was regularly discarded.

The pH of the digest was adjusted to 1·5 with hydrochloric acid and the precipitated papain and nucleic acids removed by centrifugation. Acidic polysaccharides, including chondroitin, were precipitated by the addition of 3% (w/v) Rivanol* (6:9-diamino-2-ethoxyacridine lactate monohydrate). Precipitation was complete

* Obtained from Bayer, Surbiton-on-Thames, England, under the name of "Ethodin". This compound is also variously designated: 2:5-diamino-7-ethoxyacridine lactate, "Acrinol", "Vucine", and "Acrolactine".

when the optical density at 410 mμ of the supernatant fraction, after centrifugation of the precipitate, exceeded 0·3. The precipitated polysaccharides were washed with distilled water and redissolved in 2 M ammonium formate. Diaminoethoxyacridinium ions were removed either by adsorption on alkali-washed Fuller's earth (Lloyd's reagent) or by extraction into *n*-butanol or phenoxyethanol.

Chemical analyses

Metachromatic polysaccharides were detected following high-voltage ionophoresis in carbonate buffers, pH 10·0, by immersion of dried paper strips in 0·15% (w/v) thionine in 65% (v/v) ethanol, acidified with approximately 1·0% (w/v) acetic acid. Excess stain was removed in running tap water.

Uronic acid content was determined colorimetrically with carbazole.[16] After addition of carbazole, tubes were returned to a boiling water bath for 10 min to hasten colour development. Formate ions considerably depressed colour formation. Addition of boric acid (cf. Gregory[17]) restored colour development towards normal. A final concentration of 1·5% (w/v) boric acid completely overcame the inhibition of 2 M ammonium formate.

Galactose content was determined colorimetrically by the cysteine–mannose–sulphuric acid reaction,[18] after ageing the developed coloration for 40 hr.

Total polysaccharide sulphates were determined turbidometrically on 10–100 μg samples contained in 2·5 ml of 0·8 M ammonium formate, after addition of Rivanol to a final concentration of 1 mg/ml. The optical densities of the turbidities formed were measured at 520 mμ, 20 min after the addition of Rivanol. Keratosulphate gave 70 per cent of the turbidity formed with the same amount of chondroitin sulphate. By this method, the total polysaccharide sulphates could be determined on much diluted samples of unfractionated cartilage digests. Corneal digests could not be so analysed directly for polysaccharide sulphates.

Reference materials

Chondroitin sulphate-^{35}S was isolated from calf cartilage incubated *in vitro* with sodium sulphate-^{35}S, by papain digestion and precipitation with cetyl pyridinium chloride.[15] We are indebted to Dr. J. E. Scott for this material. It was completely freed from inorganic sulphate-^{35}S by passage through a Dowex 1 column and finally precipitated with ethanol. This purified material contained 12 per cent keratosulphate (calculated from the galactose content).

Keratosulphate isolated from nucleus pulposus, contained less than 3 per cent galactosamine. Sodium chondroitin sulphate (14·2 per cent ester sulphate) isolated from calf tracheal cartilage, contained less than 1·5 per cent galactose. These polysaccharides were kindly donated by Mr. C. Antonopoulos and Dr. S. Gardell (Kemiska Laboratoriet, Serafimerlasarettet, Stockholm). Solutions of these polysaccharide sulphates were standardised by determination of their amino sugar content.[19]

EXPERIMENTAL RESULTS

Validity of the experimental methods

In order to assess the *total* sulphate-^{35}S (free and polysaccharide-bound) taken up by the incubated tissues, it was imperative to avoid losses of inorganic sulphate from the tissues after incubation. For this reason, the tissues were merely washed with

chilled Krebs–Ringer solution and counted after dehydration. Each series of incubations included at least one "contamination control" in which tissue slices were incubated with sodium sulphate-^{35}S with shaking either at 2 °C, or with added iodoacetate at 37 °C. Iodoacetate is a very potent inhibitor of the sulphate uptake by cartilage[12] and by corneas.[20] The extremely low radioactivity of cartilage slices incubated with iodoacetate (Table 1) was evidence for removal of all extraneous sul-

TABLE 1. UPTAKE OF SULPHATE-^{35}S BY CALF CARTILAGE AND CORNEA *in vitro*
(Incorporated ^{35}S measured by "direct assay". Final volume 10 ml. Cartilage incubated 2 hr, corneas incubated 5 hr.)

Addition	Temp. (°C)	Radioactivity per tissue slice Cartilage (counts/min)	Cornea (counts/min)
None	2	10	
None	37	542	1500
Iodoacetate	37	12	429
Methanol (25 μl)	37	561	1485
Methanol (50 μl)	37	530	1465
Methanol (100 μl)	37	470	
Tween 20 (2·5 mg)	37	587	1412
Tween 20 (10 mg)	37	343	

phate-^{35}S by the washing procedures employed. The association of sulphate-^{35}S with iodoacetate-poisoned corneal slices is probably a consequence of the considerable swelling and imbibition of water which accompanied incubation of these tissues. Further analyses revealed that on average, only 8 per cent of the total sulphate-^{35}S taken up by these poisoned slices was actually incorporated into the mucopolysaccharide sulphates. Table 1 also indicates the tolerance of the tissues to the amounts of Tween (200 μg/ml) and residual methanol (<0·5% v/v) present in the steroid emulsions.

A quick accurate method was required for the quantitative separation of inorganic sulphate-^{35}S from the polysaccharide sulphates-^{35}S to allow their separate estimation, following digestion and solubilization of the cartilage and corneal slices with papain. Conventional precipitants of sulphated mucopolysaccharides (cationic detergents, streptomycin, hexamminocobaltic ions) were unsuitable for this purpose, as they all failed to *completely* precipitate the sub-milligram quantities of mucopolysaccharides liberated from digested corneal slices. Mucopolysaccharides constitute only about 2 per cent of the dry weight of cattle corneas.[2, 21] It was found that chondroitin sulphate and keratosulphate at concentrations of 20–500 μg/ml were quantitatively precipitated by Rivanol. The precipitated polysaccharide sulphates were readily soluble in 2 M ammonium formate, permitting suitable aliquots to be taken for counting. Table 2 records the results of some experiments testing the suitability of Rivanol for resolving artificial mixtures of inorganic sulphate and chondroitin sulphate or keratosulphate. Chondroitin sulphate (85 μg/ml) was quantitatively precipitated. At the same concentration, at least 92 per cent of the keratosulphate was precipitated. This was considered satisfactory in view of the difficulty in precipitating keratosulphate at all with cationic detergents.[15] Inorganic sulphate was not co-precipitated with the polysaccharides.

Effect of salicylic acid and related compounds

Potassium salicylate added at 5×10^{-3} M or in higher concentrations, considerably inhibited the sulphate uptake by fresh calf cartilage and corneal slices (Table 3). Lower concentrations were not inhibitory and in many experiments, even stimulated the sulphate uptake.

TABLE 2. SEPARATION OF SULPHATE IONS AND POLYSACCHARIDE SULPHATES WITH RIVANOL

(ChS = chondroitin sulphate, measured as glucuronic acid.
KS = keratosulphate, measured as galactose.
Precipitation in volume = 1·2 ml, pH 1·5; Rivanol = 0·1 ml, 1 per cent (w/v).
Precipitates redissolved in 1·2 ml 2 M ammonium formate.)

Composition of mixture	Rivanol added	Residual supernatant O.D.*	Residual supernatant (counts/min)	Redissolved ppt. O.D.*	Redissolved ppt. (counts/min)†
100 μg.KS + $^{35}SO_4^{2-}$	−	0·112	12,050	—	—
	+	0·010	12,070	0·103	10
20 μg.KS + $^{35}SO_4^{2-}$	−	0·022	5910	—	—
	+	0·002	5895	0·023	10
100 μg.ChS + $^{35}SO_4^{2-}$	−	0·210	7555	—	—
	+	0·005	7465	0·218	17
100 μg.ChS-^{35}S	−		1500		—
	+		17		1490
10 μg.ChS-^{35}S	−		153		—
	+		10		140
Digest‡ + 50 μg.ChS-^{35}S	−		724		—
	+		4		742

* O.D. = optical density at 535 mμ (uronic acid) or 600 mμ (galactose).
† Uncorrected for self-absorption.
‡ Papain-digested cartilage.

Magnesium ions are probably essential for mucopolysaccharide biosynthesis and sulphation. Salicylic acid forms a complex with magnesium ions, albeit at rather an alkaline pH.[22] Conducting incubations in the absence of added magnesium ions or with the addition of a more potent magnesium-complexing agent, versene (ethylene-diaminetetra-acetate), failed to mimic the inhibitory action of potassium salicylate (Table 3).

A number of related aromatic compounds were tested for their effect on the sulphate uptake by cartilage slices. Only *o*-pyrocatechuate, L-thyroxine and 2:4-dinitrophenol were inhibitory (Table 4).

Effect of hydrocortisone and related compounds

Hydrocortisone and cortisone at 10^{-4} M inhibited the sulphate uptake by cartilage slices, their 21-acetates being much less inhibitory. Deoxycorticosterone was also a much weaker inhibitor. The degree of inhibition appeared to increase with increasing age of the tissues. Table 5 records the results obtained when equal volumes of the same steroid emulsion were incubated with slices of cartilage and with corneas from young and old animals.

Because it is difficult to obtain the exact age of cattle slaughtered, some attempts were made to define the age of the incubated tissues by biochemical parameters. The

proportion of keratosulphate in human, chick and invertebrate cartilage increases with age.[23, 24] Therefore the ratio of the uronic acid to the galactose contents of the Rivanol-precipitated mucopolysaccharides, was determined after digesting slices of young calf and cow cartilage and corneas with papain. A decrease in this ratio represents an increase in the proportion of keratosulphate relative to the sum of the

TABLE 3. EFFECT OF POTASSIUM SALICYLATE, VERSENE AND MAGNESIUM IONS ON SULPHATE-^{35}S INCORPORATION BY CALF CARTILAGE AND CORNEAL SLICES

(Incorporated ^{35}S = radioactivity per slice; for cartilage, determined by direct assay; for cornea, after papain digestion. Incubation period, 6 hr.)

Addition	Cartilage		Cornea	
	Incorporated ^{35}S (counts/min)	% Inhibition	Incorporated ^{35}S (counts/min)	% Inhibition*
None	1800	—	2816	—
Salicylate				
5×10^{-2} M	96	95		
5×10^{-3} M	715	60	1603	43
5×10^{-4} M	1920	−7†	2347	17
5×10^{-5} M	2033	−13†	3157	−11†
Versene				
10^{-3} M	2038	−13†		
10^{-5} M	2040	−13†		
Without Mg^{2+}	2400	−33†		
Without Ca^{2+}	1960	−9†		
Without both Mg^{2+} + Ca^{2+}	2485	−37†		
Iodoacetate				
($2{\cdot}5 \times 10^{-3}$ M)	25	(98)	1385	(51)

* Uncorrected for uptake by iodoacetate-poisoned slices.
† Actually a stimulation.

TABLE 4. EFFECT OF SALICYLIC ACID AND RELATED PHENOLS UPON SULPHATE-^{35}S INCORPORATION INTO CARTILAGE SLICES

(Incorporated ^{35}S measured by direct assay. Aromatic acids added as potassium salts. Incubation period, 6 hr.)

Addition	Concentration	Incorporated ^{35}S/slice (counts/min)	% Inhibition
None		1075	—
Salicylate	5×10^{-3} M	545	49
o-Pyrocatechuate (2:3-dihydroxybenzoate)	5×10^{-3} M	707	34
Gentisate (2:5-dihydroxybenzoate)	5×10^{-3} M	1055	2
γ-Resorcylate (2:6-dihydroxybenzoate)	5×10^{-3} M	1005	7
2:4-Dinitrophenol	5×10^{-4} M	225	79
	5×10^{-5} M	561	48
L-Thyroxine	$2{\cdot}5 \times 10^{-4}$ M	734	31
	$2{\cdot}5 \times 10^{-5}$ M	1011	6
Phenol	5×10^{-3} M	1034	4
Benzoate	5×10^{-3} M	1067	0

chondroitin sulphates A and C and chondroitin. Some other parameters related to the biological age of the tissues were also noted: water content of the fresh tissues and the relative activities of the slices tissues in taking up inorganic sulphate-^{35}S from the medium in the absence of drugs.

The concentration of the steroids (measured as Δ^4-3-ones) present in the medium after 6 hr incubation was always found to be at least 93 per cent (93–99·5 per cent)

TABLE 5. EFFECT OF AGE ON DRUG-SENSITIVITY OF SULPHATE-^{35}S UPTAKE BY CATTLE CARTILAGE AND CORNEAS *in vitro*

(Incubation period, 6 hr. Additions: steroids, 10^{-4} M; salicylate, 5×10^{-3} M; iodoacetate, 5×10^{-3} M.)

Addition	% Inhibition $^{35}SO_4^{2-}$ uptake by Cartilage		Cornea	
	Calf (%)	Cow (%)	Calf (%)	Cow (%)
Hydrocortisone				
alcohol	23	70	10	54
acetate	4	4		
Cortisone				
alcohol	29	47		
acetate	0	5		
Deoxycorticosterone	2	3		
Salicylic acid	64	72	56	62
Iodoacetate	98	98	71	78
Tissue parameters:				
Uronic acid: galactose	2·2	1·5	0·9	0·8
Dry: wet weight	23·0%	29·6%	20·1%	21·4%
^{35}S uptake by control (counts/min per slice)	2550	480	1500	840

of the initial concentration. There was evidently little or no metabolism (biological reduction) of the inhibitory steroids by the tissues during the incubation period.

No evidence was found for any drug-stimulated desulphation and depolymerization of the mucopolysaccharide sulphates in cartilage, as might be detected by reduction in the turbidities formed with Rivanol. The total polysaccharide sulphate content of slices incubated with hydrocortisone for periods up to 8 hr, was not significantly less than that of slices which had not been incubated at all or which had been incubated for the same period without hydrocortisone. There being no detectable change in the amount of mucopolysaccharide sulphates within the tissue, the only explanation for the observed reduction in the polysaccharide sulphate-^{35}S fraction as a result of drug action, is that incorporation of inorganic sulphate-^{35}S into the polysaccharides was inhibited.

Inorganic sulphate-^{35}S within the cartilage and cornea slices

The inorganic sulphate-^{35}S within the tissues is rapidly incorporated into the polysaccharide sulphates. More than 80 per cent of the total ^{35}S present within cartilage slices incubated for only 5 min with sodium sulphate-^{35}S, was precipitated with Rivanol. The proportion of polysaccharide sulphate-^{35}S increased to approximately 92 per cent of the total ^{35}S in the tissues after 30 min incubation, and to 95 per cent after 6 hr incubation.

The following experiment showed that the radioactivity of papain-digests remaining in solution after the Rivanol precipitation, was indeed intracellular inorganic sulphate-^{35}S (or very labile organic sulphate-^{35}S) and not small traces of unprecipitated polysaccharide sulphate-^{35}S. Aliquots of papain digests from a normal 6 hr incubation and from its iodoacetate-poisoned control were submitted to paper ionophoresis. The zones ahead of the foremost metachromatic material (chondroitin sulphates) were eluted. This area represents inorganic sulphate. Direct comparison of the radioactivity eluted from these zones showed that the slices from the normal incubation contained much more free (or labile) sulphate-^{35}S (270 counts/min) than the iodoacetate-poisoned control (48 counts/min).

The proportion of inorganic sulphate-^{35}S relative to the total sulphate-^{35}S (inorganic and polysaccharide-bound) within the tissues always increased when the slices had been incubated with the anti-inflammatory steroids or with uncoupling agents (Table 6). This finding supports the previous inference that under the action of the drugs,

TABLE 6. DISTRIBUTION OF INCORPORATED ^{35}S BETWEEN INORGANIC SULPHATE AND POLYSACCHARIDE SULPHATES IN CALF CARTILAGE AND CORNEA

(Incubation period, 5·5 hr.)

Addition	Cartilage (10 slices)			Cornea (8 slices)		
	Total ^{35}S (counts/min)	$^{35}SO_4^{2-}$ (counts/min)	$^{35}SO_4^{2-}$ (%)	Total ^{35}S (counts/min)	$^{35}SO_4^{2-}$ (counts/min)	$^{35}SO_4^{2-}$ (%)
None	59,500	2400	4·0	25,820	11,200	43
Salicylate (5×10^{-3} M)	28,400	1660	5·7	16,030	10,000	62
Dinitrophenol (5×10^{-4} M)	8300	1030	12·4			
Tween only	41,400	1800	4·3	26,280	11,300	43
Hydrocortisone (2×10^{-4} M)	24,100	1350	5·6	25,120	12,600	50
Cortisone (2×10^{-4} M)	21,100	1160	5·5			
Iodoacetate (5×10^{-3} M)				13,850	12,700	92

there is diminished incorporation of the inorganic sulphate-^{35}S, present within the tissues, into the mucopolysaccharide sulphates. However, the actual amount of inorganic sulphate-^{35}S in the drug-incubated tissues was always less than that in the corresponding controls.

If only the utilization of the intracellular sulphate had been inhibited, the labelling of the intracellular sulphate pool should have increased. There are two possible explanations for the observed reduction in the intracellular inorganic sulphate-^{35}S under the influence of the drugs. One explanation requires that in response to the drugs, the intracellular inorganic sulphate pool is augmented by non-radioactive sulphate so diluting the inorganic sulphate-^{35}S, and the excess sulphate is then excreted into the medium. This non-radioactive sulphate could only arise from within the tissue as the sulphate in the medium was radioactive. There was, however, no evidence of a drug-induced degradation of the polysaccharide sulphates, which might have liberated inorganic sulphate within the tissues. The other, simpler and more acceptable interpretation of the diminished labelling of intracellular sulphate, is that the drugs

inhibited the transport of sulphate ions across the permeability barriers between the medium and the tissues. This cannot be the only action of the drugs, for we always observed an increase in the *proportion* of inorganic sulphate-^{35}S in the drug-treated tissues, even though the actual *amount* was less than the inorganic sulphate-^{35}S present in tissues which had been similarly incubated without the addition of the drugs.

DISCUSSION

Connective tissue is rather sparsely populated with active and proliferating cells, by comparison with other body tissues. The rates of respiration and intermediary metabolism of the connective tissues are rather low and not easily measured experimentally. These tissues are relatively rich in mucopolysaccharide sulphates. The ready utilization of inorganic sulphate for their biosynthesis does afford one criterion of biochemical activity of the mesenchymal cells, readily measured with the availability of radioactive sulphate (cf. Aurell *et al.*[20]).

The inhibition of mucopolysaccharide sulphate biosynthesis by salicylates and by cortisone and its congeners, may actually be of little therapeutic value. It does, however, afford a useful experimental index with which to study whether or not these chemically dissimilar drugs have any common biochemical action, which might explain their similar pharmacological action in suppressing tissue inflammation.

Cartilage and cornea were chosen for this study since they are readily accessible and it is possible to prepare slices therefrom which do not vary greatly in activity between consecutive experiments. In cartilage the chondroitin sulphates predominate but the proportion of keratosulphate increases with age.[23, 24] In cornea, keratosulphate is the predominant polysaccharide.[2, 21] Thus, the cornea in some respects resembles "elderly" cartilage. In cartilage, mucopolysaccharide sulphates are major constituents (30–40 per cent dry weight); in cornea, they are only minor constituents (2–3 per cent dry weight). The two tissues are therefore somewhat complimentary. Incorporation of sulphate ions into the mucopolysaccharide sulphates of cornea has been described by a number of workers[20, 25, 26] but the possible effects of drugs and hormones upon corneal metabolism has hardly been investigated to date. Sulphate metabolism in cartilage has been studied more intensively.[27, 28]

We now have evidence that in both cornea and cartilage, salicylates and the anti-inflammatory steroids inhibit the incorporation of extra-cellular inorganic sulphate into the mucopolysaccharide sulphates of the tissues, by diminishing the uptake of sulphate ions by the tissues and also by partially inhibiting the incorporation of the intracellular inorganic sulphate into the polysaccharide sulphates (Fig. 1).

The specific biochemical action of salicylic acid

As in cartilage and cornea, the incorporation of sulphate ions into the polysaccharide sulphates of granulation tissue is effectively inhibited by salicylic acid.[29] Though granulation tissue and cornea both possess enzyme systems for sulphating phenol, neither of these tissues would sulphate salicylic acid and its salts.[29, 30] By contrast, other compounds which are biologically sulphated in the cornea and elsewhere in the animal body,[31] e.g. ethanol or phenol itself, do not inhibit incorporation of sulphate ions into the mucopolysaccharide sulphates *in vitro* when administered at the same concentrations (10^{-3} M) at which salicylic acid is an effective inhibitor. It therefore

seems quite improbable that salicylic acid exerts its inhibitory action by acting as an alternative sulphate acceptor, being itself sulphated, thereby diverting the supply of active sulphate (PAPS) from the normal sulphate acceptors (oligo/polysaccharides). Nor can the action of salicylic acid be simply explained by the complexing of magnesium ions, as our experiments have shown.

Amongst the many phenolic compounds tested in the present and in previous experiments,[11] the only powerful inhibitors of sulphate incorporation into cartilage

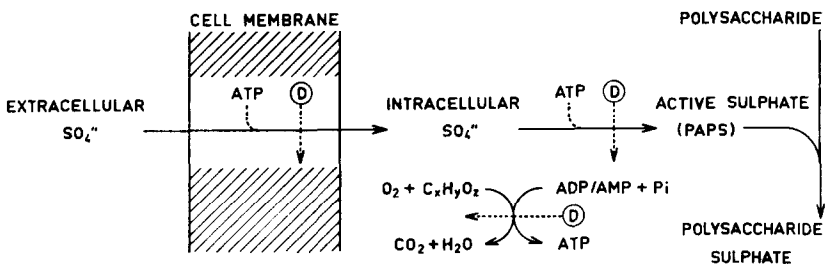

FIG. 1. Scheme depicting the probable pathway of sulphate metabolism and sites of drug inhibition (D).

were L-thyroxine and 2:4-dinitrophenol. These are both potent uncouplers of oxidative phosphorylation in mitochondria isolated from various animal tissues.[32] Salicylic acid is known to uncouple oxidative phosphorylation in rat liver and heart muscle mitochondria.[33–37] It is instructive to compare the relative potencies of dinitrophenol, thyroxine and salicylic acid in this respect. Table 7 compares the concentrations of

TABLE 7. COMPARISON OF SALICYLIC ACID WITH DINITROPHENOL AND THYROXINE (See text for references)

Compound	Equi-potent concentrations for action on		
	Cartilage	Heart mitochondria	Yeast
Salicylic acid	5×10^{-3} M	$2{\cdot}5 \times 10^{-3}$ M	8×10^{-3} M
L-Thyroxine	4×10^{-4} M	5×10^{-4} M	—
2:4-Dinitrophenol	5×10^{-5} M	$<10^{-4}$ M	10^{-4} M

these three compounds which inhibit sulphate incorporation into calf cartilage by 50 per cent and which produce the same degree of uncoupling of oxidative phosphorylation in heart mitochondria[37] and in yeast,[38] as measured by the consequent stimulation of respiration. It is yet more instructive to compare the relative activities of salicylic acid and some of its analogs, as inhibitors of sulphate incorporation into cartilage slices and as inhibitors of oxidative phosphorylation in rat liver mitochondria[33] (Table 8). The agreement between the relative potencies of these analogs acting on the two systems, is remarkable. The only exceptions (with methyl salicylate and acetyl salicylate) probably reflect the different rates of hydrolysis of these particular derivatives in cartilage and in liver mitochondrial preparations.

The stimulation of sulphate uptake by low concentrations of salicylate (5×10^{-5} M) was repeatedly observed in these experiments. It may represent chelation of some inhibitory metal ion, or possibly a "tightening" of the coupling between oxidation and phosphorylation. Such an apparent tightening has been observed with rat liver mitochondria at lower concentrations of nicotinic acid (10^{-3} M), whilst higher concentrations of nicotinic acid ($1{\cdot}5 \times 10^{-2}$ M) uncoupled oxidative phosphorylation. (Whitehouse, unpublished experiments.)

TABLE 8. RELATIVE ACTIVITIES OF SALICYLIC ACID AND ITS ANALOGUES

Compound	Relative inhibition of Sulphate uptake by cartilage I* (%)	Sulphate uptake by cartilage II† (%)	Oxidative phosphorylation in rat liver mitochondria‡ (%)
Salicylic acid	28	49	96
Methyl salicylate	15		74
Acetyl salicylate	16		72
Salicylamide	8		11
p-Aminosalicylic acid	1		3
2:3-Dihydroxybenzoic acid (*o*-pyrocatechuic acid)		34	55
2:4-Dihydroxybenzoic acid (β-resorcylic acid)	3		9
2:5-Dihydroxybenzoic acid (gentisic acid)	2	2	7
2:6-Dihydroxybenzoic acid (γ-resorcylic acid)		7	10
Benzoic acid		0	0

* From Boström and Månsson[11], with concentrations 4×10^{-3} M.
† Present experiments, with concentrations 5×10^{-3} M.
‡ From Brody[33], with concentrations 2×10^{-3} M.

The action of the steroids

There appears to be a correlation between the activity of the various steroids studied, in inhibiting sulphate uptake by mesenchymal tissues and their potency as anti-inflammatory agents.[8, 10] In both respects, cortisone and hydrocortisone (cortisol) are the most active of the natural steroids secreted by the adrenal cortex. Their respective esters and other steroid hormones (desoxycorticosterone, progesterone, etc.) were either much less active or completely inactive as inhibitors of sulphate metabolism in connective tissues. We have always found that cortisone and hydrocortisone have similar activity on three *in vitro* systems, viz. chick embryonic cartilage,[10] cattle cartilage and cattle cornea. There are, however, reports in the literature which suggest that hydrocortisone has no action on cartilage metabolism, in contradistinction to cortisone. Thus Clark and Umbreit[39] have described the inhibition of sulphate incorporation into rat xiphoid cartilage *in vitro* by cortisone and cortisone acetate and a stimulation of the sulphate uptake by unspecified concentrations of hydrocortisone and hydrocortisone acetate. For these experiments, the steroids were "dissolved" directly in the incubation medium; a procedure which we have found to be unreproducible and unsatisfactory. Another report[40] has noted differences between the action of cortisone acetate and hydrocortisone acetate on cartilage metabolism

in vivo. If indeed only the steroid alcohols are pharmacologically active, as suggested by *in vitro* studies, such discrepancies may reflect different rates of hydrolysis of cortisone acetate and hydrocortisone acetate in the whole animal, or different rates of inactivation of the steroid alcohols formed from the acetates *in vivo*.

A number of independent reports[41–46] have described the inhibition of mitochondrial oxidation by hydrocortisone and other steroid hormones when added at concentrations of the order of 10^{-4} M. There is common agreement that succinate oxidation is not inhibited and that the oxidation of other substrates mediated by DPN and the oxidation of DPNH itself is steroid-sensitive. Gallagher[46] reported that the inhibitory action of hydrocortisone on rat liver mitochondria is reversed by adding back necessary cofactors and has suggested that hydrocortisone may act as a regulator of metabolism by governing the selective semipermeability of the mitochondrial membranes. In all instances, addition of the steroids primarily affected the rates of oxidation of the various substrates (excepting succinate) by the mitochondria and not oxidative phosphorylation. Cortisone may possibly uncouple oxidative phosphorylation in liver mitochondria.[47]

Grossfeld[48] has reported some data which suggest the relevance of these observations upon isolated mitochondria, to the action of the steroids upon connective tissue. He found that hydrocortisone (3×10^{-4} M) strongly inhibited the respiration of fibroblasts in tissue culture. Fibroblasts are mesenchymal cells of the type found widely distributed throughout connective tissues. It is therefore interesting to note that desoxycorticosterone was much less active than hydrocortisone in inhibiting both fibroblast respiration[48] and sulphate metabolism in cartilage, and is almost devoid of anti-inflammatory activity.

The *net* effect of this diminished respiration (in response to the steroids) would be the same as if direct uncoupling of oxidative phosphorylation had occurred (as with the salicylates), with reduction in the amounts of ATP generated by mitochondrial oxidation.

Relationship between the biochemical and the therapeutic actions of cortisone and salicylates

Certain tissues derived from the primitive mesenchyme including connective tissue, synovia, vascular tissue and muscle are prominently involved in the so-called "collagen diseases". Inflammation of these tissues, caused by a variety of noxious agents, is strikingly modified by several drugs including the salicylates and congeners of cortisone. These particular drugs also modify the clinical course of a group of disorders of mesenchymal tissue with differing clinical manifestations and perhaps caused by different agents. The biological reactivity of the mesenchymal tissues to these agents (inflammation, tissue swelling, etc.) is depressed by the drugs. It seems reasonable to suppose that the drugs control the tissue reactivity at some fundamental biochemical level, common to a variety of animal species and to the diverse reactions elicited by a variety of injurious agents.

Adams and Cobb[49] drew attention to the possible relationship between anti-inflammatory activity and ability to uncouple oxidative phosphorylation. They noted, however, that 2:3-dihydroxybenzoic acid (*o*-pyrocatechuic acid) and dinitrophenol, though active as uncoupling agents, were without anti-inflammatory activity in the

erythrema inhibition assay. This assay may not truly represent the therapeutic value of the compound since pyrocatechuic acid is a useful anti-rheumatic drug.[50] The concentrations at which salicylic acid is a potent inhibitor of both oxidative phosphorylation and sulphate metabolism in cartilage ($2–5 \times 10^{-3}$ M) do correspond closely with the plasma salicylate levels of 20–30 mg % (ca. 2×10^{-3} M) which must be maintained for effective therapy in rheumatic fever.[51] Moses and Smith[52] presented data that seem to preclude any common action of anti-inflammatory steroids and of salicylic acid upon carbohydrate metabolism in liver and brain preparations. It is of course uncertain how relevant their observations may be to the action of the drugs on the intact connective tissues.

Though acting by different mechanisms, the anti-inflammatory steroids and salicylates may yet have a common effect by reducing ATP synthesis, thereby partially inhibiting a multitude of endergonic processes in the tissues. As a consequence, not only will biosynthetic mechanisms be inhibited but so will the ATP-dependent movements of certain metabolites, ions and water between the extracellular fluid and the interior of the tissues. These active transport mechanisms are often very sensitive to changes in the concentration of ATP within the cells.[53, 54] Smith[51] has discussed possible benefits of alterations in these active transport mechanisms in countering normal tissue swelling and inflammation elicited by noxious agents.

Our own observations indicate that both the transport of sulphate ions across the cell membranes and the (ATP-dependent) utilization of intracellular sulphate ions for sulphation of the mucopolysaccharides, were inhibited by the uncoupling agents, e.g. salicylic acid, and by the anti-inflammatory steroids. We can only explain this double action of these chemically dissimilar drugs in terms of an inhibition of the biosynthesis of ATP within the connective tissues. That the sulphation rather than the biosynthesis, of the (unsulphated) mucopolysaccharides should primarily be affected by the drugs[10] may reflect the relative availability of sulphate ions and carbohydrate precursors within the tissues subjected to the action of the drugs. Salicylates may actually increase glucose uptake by some tissues[55] and the anti-inflammatory steroids all promote gluconeogenesis. Thus the drugs may not diminish the supply of carbohydrate precursors for mucopolysaccharide biosynthesis. Any action of these drugs curtailing the active transport of sulphate ions from the extracellular fluid into the mesenchymal tissues, through limiting the supply of ATP, might become a determinant factor in controlling the eventual degree of sulphation of the biosynthesized mucopolysaccharides. Grossfeld, Meyer and their co-workers[56] have found that sulphated polysaccharides formed by skin, subcutaneous tissue and embryonic bone in tissue cultures, may be "undersulphated" and concluded that sulphation of the mucopolysaccharide molecules may indeed proceed independently of their biosynthesis.

The efficacy of some anti-inflammatory drugs may perhaps depend on the age of the target tissues. Disorders of connective tissues usually increase in severity with age and might be considered "diseases of ageing". It was therefore interesting to find that the sensitivity of elderly cartilage and cornea to cortisone and hydrocortisone was greater than that of more juvenile tissues. Whether this increase in drug-sensitivity with age rests with the declining activity of mitochondria within the tissues or is due to other physicochemical changes in the tissues with ageing (e.g. increase in keratosulphate, decrease in water content) is problematical. It should be noted that the

relative inhibition of sulphate metabolism in cartilage and the cornea by salicylic acid was rather less age-dependent.

Acknowledgements—We thank Prof. G. Biörck for his interest in this work. It is also a pleasure to acknowledge the assistance and advice we have received from Messrs. H. Johnsson and A. Vestermark, Drs. S. Gardell and L. Rodén. One of us (M.W.W.) is greatly indebted to the Empire Rheumatism Council, Great Britain, for the award of a Geigy travelling Fellowship. This investigation was supported by grants from the Helen Hay Whitney Foundation, New York, the National Institutes of Health (no. A.2568 M & N), Bethesda, and from King Gustaf the Fifth's Eightieth Birthday Fund.

REFERENCES

1. K. MEYER *Struktur und Stoffwechsel des Bindegewebes* (Edited by W. H. HAUSS and H. LOSSE) p. 12. Georg Thieme Verlag, Stuttgart (1960).
2. K. MEYER, A. LINKER, E. A. DAVIDSON and B. WEISSMANN, *J. Biol. Chem.* **205**, 611 (1953).
3. J. FRIED and A. BORMAN, *Vitam. and Horm.* **16**, 303 (1958).
4. L. L. LAYTON, *Arch. Biochem. Biophys.* **32**, 224 (1951).
5. L. L. LAYTON, *Proc. Soc. Exp. Biol., N.Y.* **76**, 596 (1951).
6. G. ASBOE-HANSEN, *Physiol. Rev.* **38**, 446 (1958).
7. H. BOSTRÖM and E. ODEBLAD, *Ark. Kemi* **6**, 39 (1953).
8. J. W. LASH and M. W. WHITEHOUSE, *Lab. Invest.* **10**, 388 (1961).
9. M. W. WHITEHOUSE and J. W. LASH, *Acta Endocrinol.* Suppl. No. 51, 957 (1960).
10. M. W. WHITEHOUSE and J. W. LASH, *Nature, Lond.* **189**, 37 (1961).
11. H. BOSTRÖM and B. MÅNSSON, *J. Pharm., Lond.* **7**, 185 (1955).
12. H. BOSTRÖM and B. MÅNSSON, *Ark. Kemi* **6**, 23 (1953).
13. H. BOSTRÖM, E. JORPES, B. MÅNSSON, L. RODÉN and A. VESTERMARK, *Ark. Kemi* **8**, 469 (1955).
14. S. GARDELL, *Ark. Kemi* **4**, 449 (1952).
15. J. E. SCOTT, *Methods of Biochemical Analysis* (Edited by D. GLICK) Vol. VIII, p. 145. Interscience, New York and London (1960).
16. Z. DISCHE, *J. Biol. Chem.* **167**, 189 (1947).
17. J. D. GREGORY, *Arch. Biochem. Biophys.* **89**, 157 (1960).
18. Z. DISCHE, L. B. SHETTLES and M. OSNOS, *Arch. Biochem. Biophys.* **22**, 167 (1949).
19. S. GARDELL, *Methods of Biochemical Analysis* (Edited by D. GLICK) Vol. VI, p. 289. Interscience, New York and London (1960).
20. G. AURELL, C-H. DOHLMAN and L. RODÉN, *Acta Opthalmol.* **34**, 281 (1956).
21. J. POLATNICK, A. J. LA TESSA and H. M. KATZIN, *Biochem. Biophys. Acta* **26**, 361 (1957).
22. C. A. ZITTLE, L. PEPPER and E. W. BINGHAM, *Arch. Biochem. Biophys.* **88**, 94 (1960).
23. K. MEYER and D. KAPLAN, *Nature, Lond.* **183**, 1267 (1959).
24. J. W. LASH and M. W. WHITEHOUSE, *Arch. Biochem. Biophys.* **90**, 159 (1960).
25. B. WORTMAN and J. L. STROMINGER, *Amer. J. Opthalmol.* **44**, 291 (1957).
26. M. SUDA, *Jap. J. Opthalmol.* **2**, 172 (1958).
27. H. BOSTRÖM, *Struktur und Stoffwechsel des Bindegewebes* (Edited by W. H. HAUS and H. LOSSE) p. 20. Georg Thieme Verlag, Stuttgart (1960).
28. H. BOSTRÖM and L. RODÉN, *The Chemistry and Biology of Compounds Containing Aminosugars* (Edited by R. W. JEANLOZ and E. A. BALAZS). Academic Press, New York (1961).
29. G. LOEWI and P. W. KENT, *Biochem. J.* **65**, 550 (1957).
30. B. WORTMAN, *Amer. J. Physiol.* **198**, 779 (1960).
31. H. BOSTRÖM and A. VESTERMARK, *Biochem. Pharmacol.* **6**, 72.
32. T. M. BRODY, *Parmacol. Revs.* **7**, 335 (1955).
33. T. M. BRODY, *J. Pharmacol. Therap.* **117**, 39 (1956).
34. I. BOSUND, *Acta Chem. Scand.* **11**, 541 (1957).
35. R. PENNIALL, *Biochem. Biophys. Acta* **30**, 247 (1958).
36. S. W. JEFFREY and M. J. H. SMITH, *Biochem. J.* **72**, 462 (1959).
37. L. PACKER, F. K. AUSTEN and E. C. KNOBLOCK, *Proc. Soc. Exp. Biol., N.Y.* **100**, 239 (1959).
38. V. MOSES and M. J. H. SMITH, *Biochem. J.* **74**, 18P (1960).
39. I. CLARK and W. W. UMBREIT, *Proc. Soc. Exp. Biol., N.Y.* **86**, 558 (1954).
40. R. LOTMAR, *Experientia* **16**, 303 (1960).

41. M. F. BIRABEN, R. LOWY and J. TRÉMOLIÈRES, *C.R. Soc. Biol., Paris* **92**, 936 (1958).
42. R. LOWY, R. DERACHE and J. TRÉMOLIÈRES, *Bull. Soc. Chim. Biol., Paris* **40**, 127, 137, 151 (1958).
43. W. WERNITSCH, H. J. HÜBENER and S. CLOEREN, *Hoppe-Seyl. Z.* **313**, 12 (1958).
44. P. K. JENSEN, *Nature, Lond.* **184**, 451 (1959).
45. K. L. YIELDING and G. M. TOMKINS, *Proc. Nat. Acad. Sci., Wash.* **45**, 1730 (1959).
46. C. H. GALLAGHER, *Biochem. J.* **74**, 38 (1960).
47. W. KERPPOLA, *Endocrinology* **67**, 92 (1960).
48. H. GROSSFELD, *Endocrinology* **65**, 777 (1959).
49. S. S. ADAMS and R. COBB, *Nature, Lond.* **181**, 773 (1958).
50. N. E. CLARKE, C. N. CLARKE and R. E. MOSHER, *Amer. J. Med. Sci.* **235**, 7 (1958).
51. M. J. H. SMITH, *J. Pharm., Lond.* **11**, 705 (1959).
52. V. MOSES and M. J. H. SMITH, *Biochem. J.* **78**, 424 (1961).
53. B. ANDERSON and H. H. USSING, *Comparative Biochemistry* (Edited by M. FLORKIN and H. S. MASON) Vol. II, p. 371. Academic Press, New York and London (1960).
54. R. WHITTAM, *Ann. Rep. Progr. Chem.* **57**. In press.
55. P. J. RANDLE and G. H. SMITH, *Biochem. J.* **70**, 490 (1958).
56. H. GROSSFELD, K. MEYER, G. GODMAN and A. LINKER, *J. Biochem. Biophys. Cytol.* **3**, 391 (1957).