Biochemical Pharmacology, 1962, Vol. 11, pp. 1175–1201. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF SOME ANTI-INFLAMMATORY (ANTI-RHEUMATIC) DRUGS ON THE METABOLISM OF CONNECTIVE TISSUES

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(*Received* 25 *May* 1962; *accepted* 19 *July* 1962)

Abstract—The following reactions in cartilage and cornea have been studied *in vitro*: (i) the incorporation of glucose-^{14}C, acetate-^{14}C and $^{35}SO_4^{2-}$ into mucopolysaccharide sulphates; (ii) the oxidation of glucose-^{14}C, acetate-^{14}C, pyruvate-^{14}C and octanoate-^{14}C.

Reaction (i) was inhibited by salicylate, many steroids, phenylbutazone, chloroquine and cinchophene. Closely related compounds, devoid of anti-inflammatory activity, did not affect this reaction. It is concluded that these drugs diminish anabolic reactions in connective tissue by inhibiting fundamental exergonic reactions.

Salicylate, phenylbutazone and cinchophene each had an immediate effect upon reaction (i). Hydrocortisone and chloroquine only manifested their effect after a significant time lag (1 to 2 hr). Inhibition by chloroquine and hydroxy-chloroquine was not reversed on removing the drugs: the action of the other drugs tested was wholly reversible. With the possible exceptions of hydrocortisone, the potency of the drugs was independent of the age of the tissues.

The relationship between structure and activity has been explored with some analogues of salicylic acid, phenylbutazone and cinchophene. Drug activity *in vitro* is correlated with lipophilic character and ability to form complexes with metal ions.

Cortisone was a less potent drug *in vitro* than either hydrocortisone or prednisolone; prednisone was inactive *in vitro*.

CERTAIN tissues derived from the primitive mesenchyme including connective tissue, synovia and vascular tissue are involved in the so-called "collagen diseases". Inflammation of these tissues caused by a variety of noxious agents, is strikingly modified by the following distinct and chemically unrelated classes of drugs:

(a) salicylic acid and certain derived *o*-hydroxybenzoates;

(b) 11-oxy-corticosteroids(pregn-4-ene-3-ones), chemically related to cortisone;

(c) phenylbutazone (butazolidine) and its metabolites;

(d) antimalarials based on 4-aminoquinoline, e.g. chloroquine (resochin).

The object of the present investigation was to determine what action, if any, these various drugs might have in common upon the cellular reactions and metabolism of mesenchymal tissues.

These tissues have rather low rates of metabolism and respiration. They contain as characteristic constituents mucopolysaccharide sulphates, including one or more of the chondroitin sulphate and keratosulphate,[1–3] which are continually synthesized by

the mesenchymal cells (fibroblasts). The utilization of inorganic sulphate (S_i)* for their biosynthesis[4] affords one criterion of biochemical activity of the mesenchymal cells.

Previous studies with $^{35}S_i$, summarized elsewhere,[5] have shown that both the anti-inflammatory steroids and salicylates inhibit the biosynthesis of mucopolysaccharide sulphates by cartilage and corneal slices *in vitro*, so reproducing one known action of these drugs *in vivo*. Analysis of this effect suggests that one primary biochemical action of the drugs is to inhibit the supply of energy for anabolic processes and for the active uptake of sulphate ions by the tissues.

This report describes experiments in which (i) the incorporation of $^{35}S_i$, acetate-^{14}C and glucose-^{14}C into polysaccharide sulphates by cornea and cartilage, and (ii) the oxidation of glucose and other substrates by cartilage, have been studied in the presence of all four classes of drugs. Some observations have been made upon the reversability of drug action *in vitro* and the relationship between drug action and chemical structure.

EXPERIMENTAL

Materials

Drugs were donated by various pharmaceutical companies. Chemical analogues were synthesized by standard procedures or if available, were obtained from commercial suppliers, principally British Drug Houses Ltd., Poole, Dorset; L. Light and Co., Colnbrook, Bucks.; Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.; and Mann Research Laboratories, New York, U.S.A. These compounds were checked for m.p. and where feasible, were re-crystallized before use. Hydroxychloroquine sulphate and chloroquine sulphate were freed of sulphate ions by extraction from alkaline solution (1 N sodium hydroxide) with methylene dichloride. The methylene chloride was evaporated in a stream of air and the free bases dissolved in aqueous NaH_2PO_4. These aqueous solutions were standardized against solutions of the freshly dissolved sulphates by measuring their optical density at 329 mμ. (The drug activity *in vitro* of chloroquine phosphate prepared in this manner was compared with that of authentic chloroquine diphosphate, "Avloclor", and found to be identical.)

Tissues

Costal, nasal and tracheal cartilage were obtained from new-born calves, from cattle aged 2 years, and older beasts (from 5 to 8 years old). Freshly excised eyes from cattle aged 2 years were brought to the laboratory packed in ice. Circular slices of costal and nasal cartilage and of cornea were prepared as described previously[5, 6] within 4 hr of slaughter. Tracheal cartilage was cut to a thickness of approximately 0·5 mm (approx. 5 mg dry weight per slice).

For any one series of incubations, slices of tracheal and costal cartilage were all prepared from one animal. Slices of cornea and nasal cartilage were prepared from the

* Abbreviations used throughout the text: S_i = inorganic sulphate; PS = (muco)polysaccharide sulphates; phenylbutazone = 1:2-diphenyl-4-*n*-butyl-pyrazolidine-3:5-dione (see Fig. 1); chloroquine = 7-chloro-4-(1-methyl-4-diethylaminobutylamino)-quinoline; hydroxychloroquine = 7-chloro-4[4-(N-ethyl-N-β-hydroxyethylamino)-1-methylbutylamino]-quinoline; Mepacrine = 3-chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)-acridine; cinchophene = 2-phenyl-quinoline-4-carboxylic acid(2-phenyl-cinchoninic acid); Rivanol (Ethodin) = 6:9-diaminoethoxyacridinium lactate; phenidone = 1-phenyl-pyrazolid-3-one; DOC = 11-deoxycorticosterone; ATP = adenosine-5′-triphosphate; DON = 6-diazo-5-oxo-L-norleucine; DMF = N,N-dimethylformamide.

tissues of several animals of approximately equal age, pooled in chilled Krebs–Ringer medium and sampled at random. The dry weight of the slices taken for parallel incubations differed by no more than 10 per cent.

Procedure

Steroids and other drugs poorly soluble in water, were dissolved in dimethylformamide (0·2 M solutions) and kept at 4 °C. For some experiments, stock solutions in propylene glycol were prepared by heating the steroid with glycol under a stream of carbon dioxide in a water bath. Stock solutions of phenylbutazone and its analogues were prepared afresh after three days, other stock solutions after three weeks. Aliquots from these solutions (usually 50 μl) were added directly to the incubation medium. The concentration of phenylbutazone and of the steroid hormones in solution was determined by measuring the u.v. absorption at 264 mμ and 246 mμ, respectively. Aqueous solutions (0·05 M) of organic acids and salts of weak bases with strong acids were prepared containing two equivalents of Na_2HPO_4. Other readily water-soluble drugs were dissolved directly in the incubation medium just before each experiment.

Tissue slices were incubated with the various drugs in 25 ml or 50 ml conical flasks containing 5 or 10 ml of a modified Krebs–Ringer phosphate medium, pH 7·4. One vol of isotonic magnesium chloride (0·11 M) was added in place of magnesium sulphate, per 100 vol of isotonic sodium chloride. Each series of incubations included at least one flask in which slices were incubated with 5×10^{-3} M sodium iodoacetate.[5] Calcium and magnesium ions were omitted from the incubation medium for those experiments with drugs precipitated by divalent cations, e.g. cinchophene, thyroxine. After pre-incubation (from 15 min to 3 hr) at 37 °C in air with shaking, sodium pyruvate-2-^{14}C (0·25 μc), sodium octanoate-1-^{14}C (0·03 μc), sodium acetate-1-^{14}C (2·5 μc), glucose-U-^{14}C (2·5 μc) or sodium sulphate-^{35}S (2·5 μc) was added in 0·5 ml of the Krebs–Ringer medium to each flask. Slices were incubated for a further period, varying from 1 to 6 hr. Respiratory carbon dioxide was trapped in 0·25 ml 2·5 N sodium hydroxide contained in the suspended centre well attached to the ground glass stopper of a 50 ml flask (i.e., "Cavett/Widmark" type flasks). The volume of liquid in the centre well at the end of the incubation was measured. Aliquots (0·1 M) were withdrawn and assayed for ^{14}C by liquid scintillation counting after dispersion in "diotol"* or toluene–ethanol (70 : 28 v/v), PPO (0·4% w/v), containing 2% (w/v) colloidal silica ("Aerosil" or "Cab-o-sil"). In all respiratory experiments, one flask containing ^{14}C-substrate but *no* cartilage slices was included in each series of incubations to measure any spontaneous decomposition or microbial oxidation of the substrate to $^{14}CO_2$. Oxidation data were discounted if oxidation by this control exceeded 20 per cent of that by a parallel incubation *with* cartilage in a drug-free medium.

At the end of the incubation period, tissue slices were washed three times with chilled isotonic chloride. Costal and nasal cartilage slices were mounted in specially recessed metal planchettes,[5] allowed to dry slowly at room temperature overnight then oven-dried at 100 °C (15 min). Washed corneal slices were incubated with shaking at 37 °C for a further 15 min, in isotonic sodium chloride–phosphate buffer, pH 6·5, to facilitate the release of extraneous $^{35}S_I$ entrained in the considerably swollen slices, washed twice further in isotonic saline, oven-dried, weighed and digested with papain.

* Composed of toluene–dioxane–methanol (10:10:6 v/v) containing 8% (w/v) naphthalene, 0·5% PPO and 0·01% POPOP.

Control experiments indicated no significant loss of ^{35}S-labelled mucopolysaccharides from the corneal slices during this short second incubation.

Measurement of incorporated radioactivity

The radioactivity of the washed and dried costal or nasal cartilage slices was first measured approximately by direct counting with an end-window Geiger–Muller tube. These slices were then digested with activated papain (see below) for a more accurate assay of incorporated radioactivity. Tracheal cartilage and corneal slices were dried at 100 °C, weighed and digested with papain. Approximately 40 mg of dried cartilage from each incubation was digested overnight at 65 °C in 0·5 ml 0·05 M sodium phosphate, pH 6·8, containing 0·005 M cysteine hydrochloride and 0·005 M disodium EDTA and the enzyme(s) extracted from 2·5 mg crude commercial papain. Cartilage slices which had been incubated with quinoline bases, e.g. chloroquine, and dried corneal slices (ca. 50 mg) were digested with twice this papain concentration. After centrifugation, 50 μl aliquots were plated on sand-blasted aluminium disks, dried at 100 °C and assayed for radioactivity with an end-window tube.

Mucopolysaccharide sulphates were precipitated from the residual digestion mixture, acidified with 0·1 ml 1 N hydrochloric acid, by the addition of Rivanol (Ethodin) sufficient to ensure a slight excess in the supernatant. (For cartilage digests 0·4 ml 4% (w/v) Rivanol; for corneal digests 0·4 ml 1·5% (w/v) Rivanol was usually sufficient.) Precipitation of the mucopolysaccharides was greatly facilitated by agitation of the tube contents with a Vortex Junior mixer (Scientific Industries Inc., Springfield, Mass., U.S.A.) 100 μl aliquots of the supernatant fraction were assayed for radioactivity as a measure of the inorganic sulphate-^{35}S ($^{35}S_i$) or glycogen-^{14}C and low molecular weight ^{14}C-compounds contained within the tissue. The radioactivity of the precipitated polysaccharide sulphates was then obtained by difference. For confirmation, the precipitated polysaccharides were redissolved in piperidine or 40% aqueous dimethyl sulphoxide and assayed for radioactivity after drying aliquots of these solutions on aluminium disks.

Chemical analyses

Cartilage slices were digested with twice crystallized papain (British Drug Houses Ltd., Poole, England). Colorimetric analyses[5] for keratosulphate and chondroitin sulphates were carried out on the acidified papain digests and also on Rivanol-precipitated polysaccharides after their resolution in 1·4 M potassium acetate. Diaminoethoxy-acridinium ions were removed before analysis by adsorption on thoroughly water and alcohol-washed sodium sulphoethyl-cellulose (Serva Entwicklungs-Labor., Heidelberg, Germany) or alkali- and water-washed magnesium silicates (Fuller's earth or "Florisil", Floridin Company, Tallahassee, Florida, U.S.A.).

Polysaccharide sulphates in papain digests were determined turbidometrically with Rivanol in the presence of 0·8 M ammonium formate as previously described,[5] after precipitating nucleic acids and residual proteins at pH 1·5 with hydrochloric acid or trifluoroacetic acid.

RESULTS

Uptake of ^{14}C and ^{35}S by cartilage and cornea

Polysaccharide sulphates containing ^{14}C were isolated from papain digests of cartilage slices, which had been incubated with glucose-^{14}C or with acetate-^{14}C in Krebs–Ringer phosphate. The acetate-^{14}C is incorporated into the acetyl group of

N-acetyl-galactosamine, present in the chondroitin sulphates. After incubation with acetate-^{14}C, the polysaccharide sulphate fraction contained ca. 20–25 per cent of the ^{14}C in the slices. After incubation with glucose-^{14}C, this fraction contained ca. 35–40 per cent of the ^{14}C content of the slices. The radioactivity of the polysaccharide sulphate fraction was retained both after removal of glycogen from the papain digests with salivary amylase and after reprecipitation from aqueous solution with alcohol or Rivanol. The same fraction was readily labelled with ^{35}S on incubating cartilage (and cornea) slices with sodium sulphate-^{35}S ($^{35}S_i$).

When tissues which had been incubated with iodoacetate (5×10^{-3} M) were digested with papain (a sulphydryl enzyme), the yield of polysaccharide sulphates (determined turbidometrically) was not significantly less than the yield from tissues incubated without iodoacetate. The radioactivity of the polysaccharide sulphate fraction isolated from iodoacetate-poisoned tissues was never more than 10 per cent of the radioactivity incorporated into this fraction in parallel incubations without iodoacetate. Tissues incubated with mercuric chloride (10^{-3} M) or *p*-hydroxymercuribenzoate (10^{-3} M) were incompletely digested by papain. Iodoacetate was therefore added to one incubation in each series of experiments to provide a "contamination control" and a measure of the radioactivity contained in the tissues, other than in the polysaccharide sulphate fraction.

Addition of sodium fumarate, acetoacetate or pyruvate (all at 10^{-3} M) to the incubation medium did not significantly stimulate uptake of $^{35}S_i$ by either corneal or cartilage slices. Preincubation with L-glutamine (10^{-4} M) for 45 min at 37 °C stimulated the uptake of $^{35}S_i$, acetate-1-^{14}C and glucose-^{14}C by cartilage (cf.[7]) and of $^{35}S_i$ by cornea. The glutamine effect on corneal metabolism was very variable. Addition of D-glucose or the glutamine antagonists, DON (5×10^{-4} M) and azaserine (5×10^{-3} M), to the medium had no effect on $^{35}S_i$ uptake by cornea. By contrast glucose stimulated, and these glutamine antagonists inhibited, $^{35}S_i$ uptake by cartilage. For routine observations, no oxidizable substrate was included in the medium. No qualitative differences were observed when these tissues were incubated with the various drugs without further addition, and when glucose or glutamine or beef serum (45 per cent by volume), singly or in combination, were included in the incubation medium.

Cartilage slices took up $^{35}S_i$ from the medium continuously during incubation at 37 °C for 6 hr or more. The uptake of $^{35}S_i$ by corneal slices at 37 °C was usually proportional to the time of incubation over a period of 3 hr. After this 3-hr period, the extent of any further $^{35}S_i$ uptake by the corneal slices varied widely from one experiment to another. For this reason it was not possible to carry out satisfactorily an extended time-study of the action of some drugs upon corneal metabolism.

Both cartilage and cornea, stored at 4 °C in closed containers for 24 hr, retained approximately 40–50 per cent of the capacity of the fresh tissues for incorporating glucose-^{14}C and $^{35}S_i$ into polysaccharide sulphates. Fresh tissues were used for all experiments with the drugs described in this paper.

Sodium octanoate-1-^{14}C was readily oxidized to carbon dioxide-^{14}C by cartilage slices. This activity was retained after storage of whole cartilage at 4 °C for 1 day. Pyruvate-2-^{14}C, acetate-1-^{14}C and lactate-1-^{14}C were less readily oxidized to carbon dioxide, presumably due to considerable dilution by endogenous lactate within the tissue. Dicarboxylic acid substrates (succinic acid-1:4-^{14}C, glutamic acid-1-^{14}C) were not appreciably oxidized.

Addition of drugs

Propylene glycol proved unsatisfactory as a general vehicle for adding drugs to the incubation medium, due to the limited solubility of some drugs, e.g. phenylbutazone, in the glycol and to the fact that glycol alone (0·5% v/v) often stimulated cartilage respiration and metabolism. N,N-dimethyl-formamide (DMF) was an exceedingly useful solvent for preparing strong stock solutions (0·2 M) of all the poorly water soluble drugs, with the exception of certain steroid-21-acetates, e.g. cortisone acetate.

TABLE 1. EFFECT OF DRUGS ON: (i) THE METABOLISM OF GLUCOSE-U-^{14}C BY COSTAL CARTILAGE
(ii) THE METABOLISM OF ACETATE-1-^{14}C BY TRACHEAL CARTILAGE
(iii) THE OXIDATION OF PYRUVATE-2-^{14}C AND OCTANATE-1-^{14}C BY TRACHEAL CARTILAGE

(Radioactivity of isolated polysaccharide sulphates (PS) and respiratory CO_2 expressed as % of controls, incubated without drugs. Incubation period, 5 hr.)

Drug	Conc. × 10^{-3}M	PS-^{14}C from glucose-^{14}C (%)	PS-^{14}C from acetate-^{14}C (%)	$^{14}CO_2$ from glucose-^{14}C (%)	$^{14}CO_2$ from acetate-^{14}C (%)	$^{14}CO_2$ from pyruvate-^{14}C (%)	$^{14}CO_2$ from octanoate-^{14}C (%)
None		100	100	100	100	100	100
Dimethylformamide	0·5%(v/v)	83	100	86	97	86	91
L-Glutamine	0·1	185	142	95	95	100	100
Salicylate	4	30	64	85	96	116	112
	1	87		110		107	
Salicylaldehyde	1	21	41	12	55	63	20
Salicylaldoxime	1	54		59			72
4-Hydroxybenzaldehyde	1	84	107	96	100	103	98
2:4-Dihydroxybenzaldehyde	1	38	66	100	103	82	150
Hydrocortisone	0·25	35	39	51	76	80	40
DOC	0·25	15	24	29	62	68	23
Compound "S"	0·25	30		38			25
Prednisolone	0·25	28		43		78	36
Phenylbutazone	0·5	33	25	108	87	122	40
Di-*p*-carboxyphenylbutazone	1·2	100	106	98	94	98	110
Antipyrine	1·2	89	100	90	88	93	112
Phenidone	0·5	36		118		105	52
Chloroquine	1	32	57	56	97	80	100
Cinchophene	1	33	59	63	92	57	140

Adding 50 μl DMF to 10 ml of the medium had very little, or no, effect on S_i metabolism. Table 1 indicates the effect of this quantity of DMF on the metabolism of glucose, acetate, pyruvate and octanoate by cartilage slices. DMF was therefore used as the preferred vehicle for bringing relatively insoluble drugs into aqueous solution. Dioxane was used with the DMF-insoluble steroid acetates. Comparative studies showed that the magnitude of the inhibitory action of hydrocortisone and of phenylbutazone on cartilage metabolism was exactly the same when these drugs were added as solution in methanol and Tween,[5] in propylene glycol and in DMF.

Effect of drugs

Slices of calf costal cartilage were preincubated for 45 min at 37 °C with the various drugs. Glucose-^{14}C or acetate-1-^{14}C was added to the medium and the tissues were incubated for a further 5 hr. The radioactivity of the respiratory carbon dioxide and of the tissue polysaccharide sulphates was then determined. Table 1 shows that sodium salicylate, hydrocortisone, phenylbutazone and chloroquine each inhibited the incorporation of glucose-^{14}C and of acetate-^{14}C into the polysaccharide sulphates *in vitro*. Chloroquine excepted, these drugs also inhibited ^{14}C-labelling of the non-polysaccharide fractions of cartilage digests.

This action of salicylate and phenylbutazone could not be attributed to inhibition of respiration, as measured by the oxidation of acetate-^{14}C, pyruvate-2-^{14}C, octanoate-1-^{14}C and glucose-U-^{14}C to carbon dioxide. Chloroquine diphosphate and steroid hormones inhibited the utilization of glucose for respiration and for polysaccharide synthesis. Cinchophene (Fig. 1), a hepatotoxic quinoline derivative formerly employed as an anti-inflammatory agent and analgesic, behaved like chloroquine in inhibiting pyruvate oxidation, glucose utilization and polysaccharide biosynthesis.

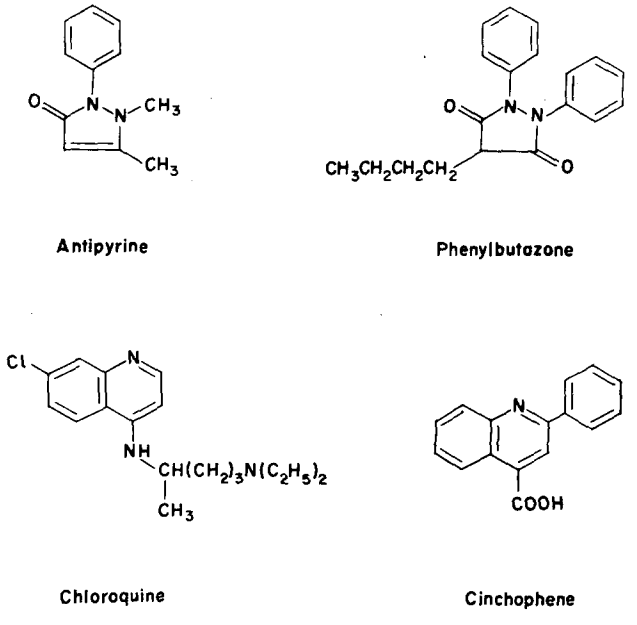

FIG. 1. Structures of some analgesic and anti-inflammatory drugs.

Compounds inhibiting the incorporation of glucose and of acetate into the polysaccharide sulphates also inhibited the incorporation of sulphate-^{35}S ions ($^{35}S_i$) into these polysulphates *in vitro* by cornea and costal, tracheal and nasal cartilage, respectively (see subsequent tables).

The following experiments showed that these drugs were truly inhibiting $^{35}S_i$ incorporation into the polysaccharides. Cartilage slices were preincubated with $^{35}S_i$ for 2 hr and then transferred to fresh incubation mediums containing one of the following drugs: hydrocortisone (5×10^{-4} M), salicylate (5×10^{-3} M), chloroquine

(10^{-3} M), phenylbutazone (10^{-3} M), but no $^{35}S_i$. After 4 hr incubation, in the presence of drugs but without $^{35}S_i$, the residual ^{35}S content of the slices and of their constituent polysaccharides was determined. In no instance was the ^{35}S content of the drug-incubated tissues found to be less than 90 per cent that of the controls, i.e. slices preincubated with $^{35}S_i$ and transferred to drug-free medium. A parallel experiment was performed with other slices from the same batch of cartilage. Slices were preincubated for 2 hr without $^{35}S_i$, then transferred to fresh medium containing each of the same four drugs and incubated for the same 4-hr period with $^{35}S_i$. The ^{35}S content of the drug-incubated tissues from this second experiment was always <60 per cent that of the controls. Together, these experiments establish that during the second incubation period when the tissues were exposed to the drugs, (i) any breakdown of polysaccharide sulphates (-^{35}S) was not significantly stimulated by the drugs, (ii) the tissues were viable and susceptible to drug action, as measured by reduced incorporation of $^{35}S_i$ into the polysaccharide sulphates.

The specificity of this drug action was delineated by incubating available chemical analogues of the anti-inflammatory drugs with slices of calf costal or young cattle tracheal cartilage and young cattle corneas in the presence of $^{35}S_i$. For these experiments, the tissues were incubated for 4 hr with $^{35}S_i$ after 15 min preincubation at 37 °C with the drug or its analogue. The incorporation of ^{35}S into the tissues and distribution of the isotope between the Rivanol-precipitable fraction (polysaccharide sulphates) and the Rivanol-soluble fraction (mainly inorganic sulphate) were then measured.

Sulphate incorporation into young cattle corneal and tracheal cartilage mucopolysaccharides was often more sensitive to the drugs, being inhibited by only one-fifth to one-half the drug concentration required to inhibit sulphate incorporation into calf costal cartilage mucopolysaccharides to a similar degree. This was not due solely to a difference in the age of animals from which the corneas and the cartilage were derived for these experiments.

Analogues of salicylic acid and thyroxine

A number of salicylate analogues have been tested previously[5, 8] for their effect, if any, upon cartilage metabolism. Only 2:3-dihydroxybenzoate (*o*-pyrocatechuate) was a significant inhibitor, though less potent than salicylate. The following compounds, inactive towards cartilage metabolism, were without significant effect (i.e., less than 15 per cent inhibition at 10^{-3} M) upon the incorporation of $^{35}S_i$ into corneal polysaccharides: sodium benzoate, 4-hydroxybenzoate, 2-methoxybenzoate, 2:4-dihydroxybenzoate (β-resorcylate), 2:5-dihydroxybenzoate (gentisate), 2:6-dihydroxybenzoate (γ-resorcylate) and 3:5-dihydroxybenzoate (α-resorcylate). In parallel incubations, 10^{-3} M salicylate, 2:3-dihydroxybenzoate and 3:4-dihydroxybenzoate (protocatechuate) inhibited sulphate incorporation into corneas by 40 per cent, 55 per cent and 20 per cent, respectively. The inhibitory action of these and other *ortho* dihydroxy-benzoates might be associated with their instability in solution and auto-oxidation during the period of incubation with the tissues. Sodium gallate (3:4:5-trihydroxybenzoate) and 2:3:4-trihydroxybenzoate were potent inhibitors of corneal sulphation (less active towards cartilage). Sodium 2:4:6-trihydroxybenzoate and 5-sulphosalicylate had no inhibitory effect. Tissue slices were considerably discoloured after incubation with the *ortho*, di- and trihydroxy acids.

2-Hydroxy *m*-toluate (*o*-cresotate) and 5-nitrosalicylate were somewhat more effective inhibitors of S_I incorporation into cartilage polysaccharides than equivalent concentrations of salicylate. 3-Nitrosalicylate and *o*-nitrophenol had no inhibitory effect. *p*-Nitrophenol was a more potent inhibitor than 5-nitrosalicylate.

The following salicylate analogues were much more potent than salicylate in their effect upon polysaccharide biosynthesis in both cartilage and cornea: 1-hydroxy-2-naphthoate, salicylaldehyde and its oxime (Tables 1 and 2). Phenol which had no action

TABLE 2. EFFECT OF SALICYLATE, SALICYLALDEHYDE, THYROXINE AND RELATED COMPOUNDS UPON THE INCORPORATION OF $^{35}S_I$ INTO POLYSACCHARIDE SULPHATES (PS) BY CARTILAGE AND CORNEA

Compound added	Conc. $\times 10^{-3}$M	Inhibition of PS-^{35}S cornea (%)	Inhibition of PS-^{35}S cartilage (%)	$^{35}S_I$/total ^{35}S cornea (%)	$^{35}S_I$/total ^{35}S cartilage (%)
None		0	0	46	5·3
Salicylate	2·5		41		9·2
	1	45	20	54	8·0
1-Hydroxy-2-naphthoate	1	87	75	85	11·0
2-Hydroxy-3-naphthoate	1	85	82	85	13·0
Salicylaldehyde	1	95	80	95	4·6
Salicylaldoxime	1		45		5·0
4-Hydroxybenzaldehyde	1	5	5	<40	5·0
2:4-Dihydroxybenzaldehyde	1	80	72	80	10·0
2:4-Dimethoxybenzaldehyde	1	<30	0		
L-Thyroxine	0·25	52	36	60	3·8
D-Thyroxine	0·25	27	20	51	4·5
L-3:3:5-Tri-iodothyronine	0·25	75	72	68	6·0

on cartilage metabolism[5] was an effective inhibitor of polysaccharide sulphation in the cornea at 10^{-3} M. Salicyl alcohol (saligenin), salicylamide, 4-hydroxybenzyl alcohol, *o*-hydroxyacetophenone, β-naphthoate, β-naphthol and 2-hydroxy-6-naphthoate were almost devoid of inhibitory activity towards cartilage (all at 4×10^{-3} M) but manifested phenol-like activity in inhibiting corneal polysaccharide sulphation. In contrast to salicylamide, salicylanilide, salicylhydroxamate and salicylhydrazide were all moderate inhibitors of cartilage sulphation.

Salicylaldehyde and a number of *ortho*-hydroxy-arylaldehydes tested were striking inhibitors of sulphate incorporation. Unlike salicylic acid, these aldehydes did not consistently "uncouple" polysaccharide sulphation from $^{35}S_I$ uptake by the tissue as measured by an increase in the ratio, $^{35}S_I$: total ^{35}S. 2:4- and 2:5-dihydroxybenzaldehydes and 2:4:6-trihydroxybenzaldehyde were active inhibitors but less potent than salicaldehyde itself. The related di- and tri-hydroxybenzoates are devoid of activity.[5] 2-Hydroxy-3-methoxy-benzaldehyde (*o*-vanillin), 2-hydroxy-1-naphthaldehyde and 2-hydroxy-3-naphthaldehyde were the most potent of the salicylate analogues examined. Approximately 50 per cent inhibition of sulphate incorporation into tracheal cartilage polysaccharides were discerned with 2×10^{-5} M *o*-hydroxynaphthaldehydes, with 10^{-4} M *o*-hydroxynaphthoates, with 2×10^{-4} M salicylaldehyde and with 2×10^{-3} M salicylate. The following aldehydes were without significant effect on

cartilage sulphation at 10^{-3} M and were very much weaker inhibitors than salicylaldehyde of the phenol-sensitive corneal sulphate metabolism: benzaldehyde, anisaldehyde, vanillin, veratraldehyde, 2:4-dimethoxybenzaldehyde, 3- and 4-hydroxybenzaldehydes. Cinnamaldehyde, protocatechuicaldehyde (3:4-dihydroxybenzaldehyde) and 2:4-dinitrobenzaldehyde each inhibited cartilage polysaccharide sulphation at 10^{-3} M. Pyridoxal hydrochloride and pyridoxal-5-phosphate were also inhibitory but very much less potent than salicylaldehyde. Pyridoxine hydrochloride had no effect upon polysaccharide sulphation *in vitro*.

L-Thyroxine inhibits polysaccharide sulphation in cartilage.[5] L-Tri-iodothyronine was rather more potent, whilst D-thyroxine was a less effective inhibitor than L-thyroxine in diminishing polysaccharide sulphation in cartilage and cornea (Table 2).

Analogues of cortisone

The following drugs, known to be systemically active glucocorticoids and anti-inflammatory agents, all inhibited sulphate incorporation into corneal and cartilage polysaccharides when added to the incubation medium at $2{\cdot}5 \times 10^{-4}$ M: corticosterone, hydrocortisone (cortisol), cortisone, prednisolone, dexamethasone, triamcinolone base (21-alcohol) and its acetonide. No general relationship was observed between the relative activities of these compounds *in vitro* and their relative potencies as anti-inflammatory drugs *in vivo*. Triamcinolone acetonide was much more potent *in vitro* than triamcinolone alcohol. Hydrocortisone-, cortisone- and prednisolone-21-acetates and triamcinolone-16:21-diacetate were all inhibitory but less active than the parent alcohols. By contrast, the following readily water-soluble steroid esters (at 10^{-3} M) had no effect on sulphate incorporation by either cornea or cartilage: cortisone-21-sulphate, hydrocortisone-21-phosphate and -21-hemisuccinate. Several samples of prednisone, obtained from three different commercial sources and checked for authentic m.p. (232° uncorr.), were all without effect on sulphate incorporation by cartilage in curious contradistinction to prednisolone.

Other steroid hormones with little or no glucocorticoid/anti-inflammatory activity in the whole animal, also inhibited both the incorporation of $^{35}S_I$ and glucose-^{14}C into mucopolysaccharides and the oxidation of glucose and pyruvate (Table 1). This series of steroids included progesterone (at 7×10^{-5} M), 11-desoxycorticosterone (DOC, cortexone), 11-desoxycortisol (Reichstein's Compound S), testosterone and adrenosterone, all at $2{\cdot}5 \times 10^{-4}$ M (see Table 5). Inhibition by these latter steroids was not modified when beef serum was added to the incubation medium, or when the drugs were added in a Tween emulsion,[5] or from propylene glycol solutions, instead of from solution in dimethylformamide. The previous report that DOC is very much less active than hydrocortisone as an inhibitor of polysaccharide sulphation must be withdrawn.*

Measurements of the optical density at 246 mμ indicated that some DOC and compound "S" was lost from solution (metabolized?) after incubation for 5 hr at 37 °C with cartilage slices; the losses being of the order of 20 per cent and 8 per cent respectively from solutions of initial concentration $2{\cdot}5 \times 10^{-4}$ M. Emulsions of these two steroids incubated without cartilage at 37 °C or stood for 24 hr at room temperature showed no decline in optical density. The optical densities of solutions of

* These experiments were conducted in 1961, 12 months later than those reported previously.[5] The sensitivity of costal cartilage metabolism to salicylate and to high doses of benzoate (5×10^{-3}M) was also notably higher in 1961 than previously.[5]

hydrocortisone, cortisone, prednisolone and dexamethasone were unchanged after incubation with cartilage and on standing for 24 hr.

Phenylbutazone (butazolidine) and related pyrazoles (Table 3)

Phenylbutazone was a potent inhibitor at 5×10^{-4} M of $^{35}S_i$ incorporation and glucose-^{14}C incorporation into polysaccharide sulphates. This particular concentration is close to the plasma level of 100 mg/l. ($3{\cdot}3 \times 10^{-4}$ M), required for anti-inflammatory activity in man.[9] The monohydroxy (phenol) derivative, oxyphenbutazone (1-phenyl-2-(4′-hydroxyphenyl)-4-*n*-butylpyrazolidine-3:5-dione, G.27202, "Tanderil") and the monocarboxy derivative (1-phenyl-2-(4′-carboxyphenyl)-4-*n*-butyl-pyrazolidine-3:5-dione, HP 433) were less active than phenylbutazone. The dicarboxy derivative (1:2-di-(4′-carboxyphenyl)-4-*n*-butyl-pyrazolidine-3:5-dione, HP 358) had no effect on the uptake of $^{35}S_i$ at 10^{-3} M. This compound is a very much less potent anti-inflammatory drug than phenylbutazone.[10] The related "salicyl analogue" (1:2-di-(3′-hydroxy-4′-carboxyphenyl)-4-*n*-butyl-pyrazolidine-2:5-dione, HP 361) at 10^{-3} M partly inhibited $^{35}S_i$ uptake by corneal slices. As already described, corneas are very sensitive to low concentrations of phenols. Thus, it seems reasonable to suppose that both HP 358 and HP 361 could penetrate into the tissues.

TABLE 3. EFFECT OF PHENYLBUTAZONE AND ITS ANALOGUES ON THE INCORPORATION OF $^{35}S_i$ INTO POLYSACCHARIDE SULPHATES (PS) BY CARTILAGE AND CORNEA.
(Drugs were tested at 10^{-3}M on cartilage; 5×10^{-4}M on cornea)

Compound added	Inhibition of PS-^{35}S		$^{35}S_i$/total ^{35}S	
	cornea (%)	cartilage (%)	cornea (%)	cartilage (%)
None	0	0	58	5·5
Phenylbutazone	80	87	85	14·0
Oxyphenbutazone	55	72	76	10·5
Sulphinpyrazone	45	78	65	10·2
Dicarboxy-phenylbutazone	0	0	56	5·5
Antipyrine	0	0	57	5·5
4-Dimethylamino-antipyrine	0	2	50	5·0
4-Aminoantipyrine	0	5	55	6·0
1-Phenyl-5-methyl-pyrazol-3-one	40	29	50	5·4
1-Phenyl-3-methyl-pyrazol-5-one	30	36	71	5·5
Picrolonic acid	65	61	66	6·8
1:3-Diphenylpyrazol-5-one	70	86	72	14·0
1-Phenyl-pyrazolid-3-one	90	94	72	13·0

Analogues of phenylbutazone in which the 4-butyl group is replaced by the *n*-propyl group (HP 482) and the 2-phenylsulphoxyethyl group (sulphinpyrazone, G 28315, "Anturan") respectively, also inhibited $^{35}S_i$ incorporation but were less active than phenylbutazone itself. The 4-hydroxy analogue and phenylbutazone-3-methyl (enol) ether (1:2-diphenyl-3-methoxy-4-butyl-pyrazol-3-ene-5-one) had no effect upon $^{35}S_i$ incorporation. Neither of these compounds exhibit anti-inflammatory activity in conventional assays.[11] Other 4-butyl-pyrazolidine-3:5-diones (HP 434, HP 435), known to be inactive as anti-inflammatory agents,[10] were without effect on polysaccharide sulphate synthesis *in vitro*.

Non-enolizable pyrazolones, commonly employed as analgesics and anti-pyretics, such as antipyrine (phenazone), 4-dimethylamino-antipyrine (dipyrine, pyramidone) and 4-amino-antipyrine had no detectable effect on the biosynthesis of polysaccharide sulphates and cartilage respiration. By contrast, the following pyrazolones all inhibited the incorporation of $^{35}S_i$ into polysaccharide sulphates: 1-phenyl-5-methyl-pyrazole-3-one, 1-phenyl-3-methyl-pyrazole-5-one, 1-*p*-nitrophenyl-3-methyl-4-nitro-pyrazole-5-one (picrolonic acid), 1-phenyl-pyrazole-5-one-3-carboxylic acid, 1-phenyl-pyrazolid-3-one ("Phenidone") and 1:3-diphenyl-pyrazole-5-one (Table 3). All these ketones are without a substituent at N_2. They must therefore be in tautomeric equilibrium with the corresponding enols, i.e. 3- or 5-hydroxy-pyrazoles. Phenidone (which is used commercially as a photographic developer) is presumably readily oxidized to the corresponding pyrazolone.

Non-ketonic pyrazoles such as 1-phenyl-pyrazole and keto-imidazoles (e.g. 5-phenyl-hydantoin) had no effect upon sulphate incorporation.

Those compounds which were the most potent inhibitors of polysaccharide sulphate biosynthesis (phenylbutazone, phenidone, diphenyl-pyrazolone) increased the *proportion* of inorganic sulphate ($^{35}S_i$) in the incubated cornea and cartilage slices, even though the actual *level* of $^{35}S_i$ was always less than in the drug-free controls.

TABLE 4. EFFECT OF CHLOROQUINE, CINCHOPHENE AND THEIR ANALOGUES ON THE INCORPORATION OF $^{35}S_i$ INTO POLYSACCHARIDE SULPHATES (PS) BY CARTILAGE AND CORNEA
(Drugs were tested at 10^{-3}M on cartilage; 6×10^{-4}M on cornea)

Compound added	Inhibition of PS-^{35}S		$^{35}S_i$/total ^{35}S	
	cornea (%)	cartilage (%)	cornea (%)	cartilage (%)
None	0	0	48	5
Chloroquine	42	74	57	34
Santoquin*	79	92	79	46
Mepacrine	75	86	82	40
Chlorothiazide	8	4	45	6
Quinoline	5	0	46	5
2-hydroxyquinoline	28	8	62	5
8-hydroxyquinoline	82	55	85	23
Quinine	25	47	51	8
Diethylaminoethanol	0	0	45	5
Promethazine	75	95	60	9
Cinchophene	72	67	73	25
Isonicotinic acid	6	6	45	5
2-hydroxy-cinchoninic acid	16	15	54	5

* Tested at 5×10^{-4}M on cartilage.

Chloroquine, cinchophene and their analogues

Chloroquine diphosphate (Nivaquin B, Sanoquin, Resochin), its 3-methyl(quinoline) derivative, Santoquin (Nivaquin C) and its methoxyacridine analogue, Mepacrine (Atebrin, Quinacrine) were all potent inhibitors of polysaccharide sulphate biosynthesis (Table 4). Hydroxychloroquine (Plaquenil) was approximately 90 per cent as potent as chloroquine at 10^{-3} M.

Papain digestion of the drug-incubated tissues and liberation of the mucopoly saccharides therefrom was much retarded by the considerable binding of the bases in the tissue slices. Increasing either the quantity of papain (see experimental methods) or the salt concentration in the papain-digestion mixture (from 0·05 M to 0·5 M) overcame this effect. After digestion under these modified conditions, the yield of polysaccharides from the drug-incubated slices was exactly that from tissues not incubated with any drug. Both the *proportion* of radioactivity within the tissues present as inorganic sulphate ($^{35}S_i$) and actual *amount* of $^{35}S_i$ in the tissues were increased by these drugs (Table 4). These two effects, i.e. diminished labelling of the polysaccharide sulphates and increased S_i within the tissue, were clearly discernible even at rather low concentrations of these drugs (10^{-4} M), acting on cartilage. A somewhat similar dissociation of ^{14}C uptake from ^{14}C incorporation into polysaccharides was observed on incubating cartilage slices with acetate-^{14}C. The ^{14}C content of the non-polysaccharide fraction in chloroquine-incubated slices was the same as that of the controls, but polysaccharide-^{14}C was much lower than in the controls (Table 1).

Diethylamine, diethylaminoethanol and dimethylamino-antipyrine, alone or together with quinoline, at 10^{-3} M had no effect on sulphate incorporation. Certain organic bases, containing two of the N functions of chloroquine within one molecule (e.g. chlorpromazine, promethazine, quinine, Rivanol) were strong inhibitors of polysaccharide sulphation. Unlike chloroquine they did *not* increase the $^{35}S_i$ content of the tissues but depressed it below the level of $^{35}S_i$ in the drug-free controls. The proportion of $^{35}S_i$ to total ^{35}S content in the tissues was accordingly much less with these difunctional bases than in tissues incubated with chloroquine, hydroxychloroquine, santoquin or mepacrine (Table 4). Promethazine dramatically inhibited (>80 per cent) pyruvate and octanoate oxidation in cartilage.

8-Hydroxyquinoline (oxine) and cinchophene each simulated chloroquinine in strongly inhibiting polysaccharide sulphation with concomitant increase in the S_i content of the tissues. Some readily available analogues of these two latter quinoline derivatives were also tested, e.g. 2-hydroxyquinoline (carbostyril), isonicotinic acid, 2-hydroxy-cinchoninic acid. At 10^{-3} M, none of these analogues exhibited the chloroquine-like activity of oxine and cinchophene. Oxine did not inhibit pyruvate oxidation in cartilage.

Miscellaneous compounds

Sodium aurothiomalate, which is used topically for treatment of arthritis, had little or no effect upon sulphate metabolism by calf costal cartilage at $2·5 \times 10^{-3}$ M. Inhibition of S_i incorporation into the polysaccharides was only observed when the incubation period exceeded 4 hr (e.g. 15 per cent inhibition after 5 hr with 4×10^{-3} M aurothiomalate). There was a corresponding depression of $^{35}S_i$/total ^{35}S within the tissue. Since aurothiomalate is precipitated by calcium ions, the incubation medium for these experiments contained no added calcium or magnesium ions. It was noticed that the cartilage slices suffered much "roughening" in the absence of calcium ions, enhanced by a prolonged incubation period. Adding ethylene diaminetetra-acetate (EDTA) to this calcium-free medium inhibited S_i incorporation into cartilage slices, similarly to aurothiomate (ca. 20 per cent inhibition with 2×10^{-3} M EDTA) and caused pronounced distortion and roughening of the tissue slices. It is concluded that the (feeble) inhibitary action of aurothiomalate is due in part to accelerated degeneration

of the tissue slices, possibly associated with loss of calcium ions. By contrast sodium thiomalate was a potent inhibitor of $^{35}S_i$ incorporation (45 per cent at 10^{-3} M). Other lipophilic thiols tested (cysteamine, thiosalicylate) were also inhibitors of sulphate incorporation into cartilage and corneal polysaccharides. Cysteine and glutathione were inactive at this concentration (10^{-3} M).

Sodium cinnamate, which like salicylate and thyroxine non-competitively inhibits succinic dehydrogenase,[12] was a slightly more potent inhibitor of $^{35}S_i$ and glucose-^{14}C incorporation than sodium salicylate at $2{\cdot}5 \times 10^{-3}$ M. Like salicylate, cinnamate increased the proportion of $^{35}S_i$/total ^{35}S within the tissue. Cinnamaldehyde was a more potent inhibitor than cinnamate but did not increase the $^{35}S_i$/total ^{35}S ratio. Hydrocinnamate, which is a much weaker inhibitor of succinic dehydrogenase,[12] was also a much weaker inhibitor of sulphate incorporation. 2-Hydroxycinnamate (coumarate) was a more potent inhibitor than cinnamate or salicylate. Other hydroxy-acids tested (ferulic, melilotic and 4-hydroxycinnamic) were less active than 2-hydroxy-cinnamate. Phenylpyruvate and coumarin had no effect on $^{35}S_i$ incorporation at $2{\cdot}5 \times 10^{-3}$ M. Ascorbate (10^{-3} M), α:α'-dipyridyl (5×10^{-4} M), colchicine (2×10^{-3} M), ouabain (10^{-3} M), ferrous ions (5×10^{-5} M) and cupric ions (5×10^{-5} M) each had no effect on sulphate incorporation. Cupferron and *o*-phenanthroline (5×10^{-4} M) were moderate inhibitors and uncoupled $^{35}S_i$ uptake from incorporation into polysaccharides. *p*-Acetamidophenol, the biologically active metabolite formed from the analgesics, acetanilide and phenacetin,[13] and like phenidone a photographic developer, had no inhibitory action on cartilage metabolism at 2×10^{-3} M.

TABLE 5. RELATIONSHIP BETWEEN THE INHIBITION OF ^{35}S INCORPORATION INTO CARTILAGE POLYSACCHARIDES AND LENGTH OF INCUBATION WITH DRUGS *in vitro*

(^{35}S incorporation by controls incubated with $^{35}S_i$ for the same period without drugs = 100).

Drug	Conc. $\times 10^{-3}$M	PS-^{35}S as % controls after incubation for 1 hr	2 hr	3 hr	4 hr	5 hr
None		100	100	100	100	100
Salicylate	5	35	39	34	40	32
Phenylbutazone	1	34	27	26	27	21
Chloroquine	1	105	83	73	67	60
Hydroxychloroquine	1	100	90	78	70	68
Cinchophene	2	40	35	33	36	36
Hydrocortisone	0·25	100	89	85	79	74
Cortisone	0·25	84	77	73	80	92
DOC	0·25	93	82	80	50	45
Dexamethasone	0·25	95	90	81	84	70
Prednisolone	0·25	72	65	60	58	67

Studies on the time course of drug action

Cartilage and corneal slices were preincubated with drugs for 5 min only before adding $^{35}S_i$. They were then incubated with the radioisotope and the drug for varying periods of time (from 1 to 6 hr). Tissues were also incubated with $^{35}S_i$ with no drug present for the same time intervals, to serve as controls. The incorporation of ^{35}S into the tissue polysaccharides by drug-incubated tissues and the control was then compared. Table 5 records the inhibition of ^{35}S incorporation into cartilage

mucopolysaccharides by the various drugs, observed after incubation for various time intervals.

In another series of experiments, tissue slices were preincubated with the various drugs for periods up to 4 hr before adding $^{35}S_i$. The tissues were then further incubated for only 1 hr with both the drugs and the $^{35}S_i$. As controls, tissues were preincubated for the same time period without drugs and then with $^{35}S_i$ for 1 hr only. Fig. 2 depicts the relationship between the length of the preincubation period and the incorporation of ^{35}S into the cartilage polysaccharides, expressed as percentage of the controls, in the presence of some of the drugs.

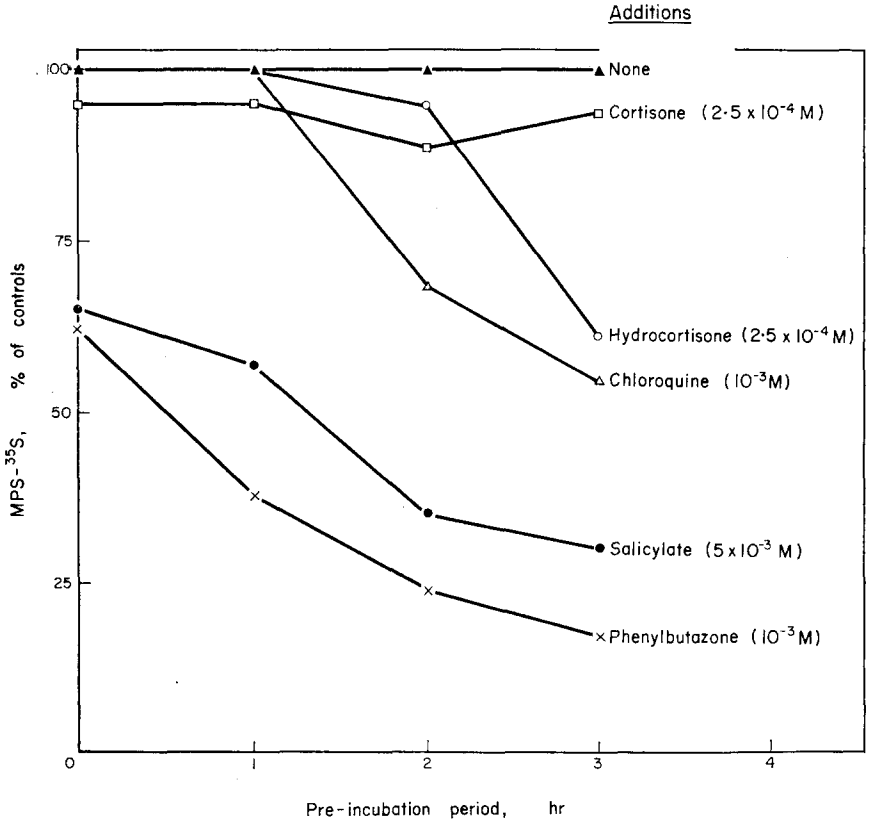

FIG. 2. Relationship between time of preincubation with a drug and incorporation of $^{35}S_i$ into, cartilage mucopolysaccharides over a fixed time period (1 hr).

Both series of experiments indicated that salicylate, phenylbutazone and cinchophene were effective immediately in inhibiting sulphate metabolism of cartilage and that chloroquine, hydroxychloroquine and hydrocortisone only inhibited sulphate metabolism after a time lag (of up to 2 hr).

Essentially, the same results were obtained with corneal slices. The inhibitory effect of phenol on corneal metabolism was manifested immediately, resembling the action of salicylate on both cartilage and cornea. Potent analogues of salicylate, e.g. 2:4-dihydroxybenzaldehyde, *o*-hydroxynaphthoates, were also immediate inhibitors of

S_i uptake by cartilage. The magnitude of the inhibitory effect of any one drug tended to increase with the length of the incubation period, with the notable exception of cortisone. It was repeatedly observed that *only* in short term incubations was cortisone a more effective inhibitor than hydrocortisone and other 11-β-hydroxysteroids. Attempts to compare the relative effects of prednisone (Δ'-cortisone) and prednisolone (Δ'-hydrocortisone) with time were unsuccessful because prednisone had no significant effect on $^{35}S_i$ incorporation *in vitro*.

The $^{35}S_i$ content of cartilage slices incubated with chloroquine diphosphate and $^{35}S_i$ was very much increased after one hour's incubation, being approximately 25 per cent of the total ^{35}S present in these tissues (compared with less than 10 per cent in the controls). Over this time interval no inhibition of ^{35}S incorporation into the polysaccharides was ever observed. These observations suggest that chloroquine may have two different actions on cartilage metabolism, one of which is relatively rapid and the other is delayed.

Studies on the reversability of drug action

Cartilage and corneal slices were preincubated with various drugs for a sufficient length of time (up to 3 hr) to exceed the lag period in drug action (see previous section). The slices were then rapidly washed three times with 0·15 M sodium chloride and finally once with the Krebs–Ringer phosphate medium. One half of the slices was transferred to fresh incubation medium containing the same concentration of drug with which the slices had been preincubated (Series A). The other half of the slices was transferred to fresh Krebs–Ringer medium containing no drugs (Series B). Both slices were then incubated with $^{35}S_i$ for periods of from 1 to 3 hr. Incorporation of ^{35}S into polysaccharides by tissues in series A and B was compared with that by the controls. These controls were slices preincubated without drugs for the same time interval, then subjected to the same washing procedure and change of medium and finally incubated with $^{35}S_i$. Some results obtained with calf costal cartilage are recorded in Table 6. Essentially identical results were observed when tracheal cartilage was used.

It is evident that only chloroquine and hydroxychloroquine had an irreversible effect upon cartilage metabolism. The inhibitory action of the other drugs, including the slow acting steroids, was readily reversed with their removal (or dilution *in situ*) on washing the tissues. The apparent stimulation of $^{35}S_i$ incorporation over and above that in the washed controls on removing salicylate and phenylbutazone, is in accord with previous observations[5] that low concentrations of salicylate (5×10^{-5} M) actually increase the incorporation of S_i into cartilage polysaccharides.

Much less complete, or even no, reversal of drug action was observed on washing preincubated corneal slices and changing the medium. This must be partly attributed to considerable retention of drug (and medium) within the considerably swollen slices. The sometimes extensive decline in metabolic activity of corneal slices after the lengthy preincubation period required with some drugs, e.g. hydrocortisone, also hindered attempts to study the reversibility of drug action using corneas.

Possible effects of age

Previous studies indicated that the inhibitory effect of hydrocortisone and cortisone upon S_i metabolism might be greater with cartilage taken from an older animal than with similar cartilage preparation from a young animal.[5] These observations had been

TABLE 6. EFFECT OF REMOVING THE DRUG (AFTER A PREINCUBATION PERIOD) ON THE DRUG ACTION

Cartilage slices preincubated with drug at 37 °C, washed then: series A, incubated 1 hr with $^{35}S_i$ in the continued presence of the drug. Series B, incubated 1 hr with $^{35}S_i$ without the drug. Drug action measured as inhibition of $^{35}S_i$ incorporation into cartilage polysaccharides (PS).

Drug	Conc. $\times 10^{-3}$M	Preincubation period (hr)	PS-^{35}S as % controls series A	series B
(None)				(100)
Salicylate	5	2	34	118
Cinnamate	2·5	2	70	103
Phenylbutazone	0·5	2	25	114
Phenidone	0·5	2	69	92
Chloroquine	1	2	82	87
		3	50	56
Hydroxychloroquine	1	2	72	68
		3	57	60
Quinine hydrochloride	1	2	55	87
Cinchophene	1	2	40	108
Hydrocortisone	0·25	2	100	
		3	64	92
DOC	0·25	2	61	104
Prednisolone	0·25	2	71	100
Dexamethasone	0·25	2	88	106

made with costal cartilage which can only be obtained from cattle aged less than 3 yr. This tissue is almost completely ossified in older beasts.

Table 7 indicates the relative potency of the drugs as inhibitors of S_i metabolism when incubated with slices of tracheal and nasal cartilage obtained from new-born

TABLE 7. DRUG INHIBITION OF $^{35}S_i$ INCORPORATION BY TRACHEAL, NASAL AND COSTAL CARTILAGE FROM NEW-BORN CALVES AND ELDERLY CATTLE

(Cattle were > 5 years old. Cartilage slices incubated *in vitro* for 5 hr).

Drug	Conc. ($\times 10^{-4}$M)	PS-^{35}S as % drug-free controls Calf tracheal (%)	Old tracheal (%)	Calf nasal (%)	Old nasal (%)	Calf costal (%)
None		100	100	100	100	100
Salicylate	50	13	32	65	22	40
	5	76	75	93	78	
Hydrocortisone	2·5	77	65	68	54	77
	0·5	93	110	118	104	
Chloroquine	9	50	75	82	69	73
	0·9	100	100	96	100	95
Phenylbutazone	8	25	35	31	17	25
	1	80	80	72	71	89
Parameters of age (see text)						
Metabolic index		0·54	0·35	0·74	0·40	1·0
Dry: wet weight (%)		24·5	30·1	17·2	23·4	23·1
Uronate: galactose		7·0	4·2	22	9·2	

calves and from cattle aged from 6 to 8 yr old. For comparison, the potency of the same drug concentrations acting on calf costal cartilage, is recorded here also. The relative metabolic index of the different cartilages *in vitro* was gauged very approximately by computing the ^{35}S incorporation into polysaccharides in the drug-free controls, per mg dry weight of slices, during a 4 hr incubation period in the presence of 2·5 μc $^{35}S_i$; the activity of calf costal cartilage being assigned a value of 1·0. The decline in this index with increased age observed in these and other [5, 6] experiments affords one parameter of ageing in cartilage, which parallels the known decline with age of cartilage respiration.[14] Further parameters[5] were provided by the dry : wet weight ratios of the freshly cut slices and the ratio of chondroitin sulphates (as glucuronic acid) to keratosulphate (as galactose) in the Rivanol-precipitated polysaccharides, following digestion with twice crystallized (and galactose-free) papain.

These drugs had similar effects upon ^{35}S metabolism in the three different types of cartilage. No general increase in drug-sensitivity of the tissue with age could be discerned in these experiments. Elderly tracheal and nasal cartilage appeared to be more susceptible to a given concentration of hydrocortisone than the younger tissue. A similar observation was made in experiments with costal cartilage and cornea.[5]

Miscellaneous properties of the anti-rheumatic drugs

Szent-Györgyi has propounded a theory that many compounds, including anti-rheumatic drugs, owe their pharmacological properties to an ability to participate in charge transfer reactions with normal body constituents. Using the techniques described by Szent-Györgyi[15], no evidence was found for charge transfer phenomena between those non-steroid drugs which specifically inhibit S_i metabolism and the following compounds: nicotinamide–adenine–dinucleotide (DPN), riboflavine mono phosphate (FMN), and iodine is aqueous potassium iodide (Lugol's solution).

The strong light absorption in the spectral range, 255–265 mμ, of aqueous solutions (pH 7) of phenylbutazone and its inhibitory analogues, was quenched at pH 2 and only slightly increased (<15 per cent) at pH 12. These drugs must therefore react at physiological pH as the almost wholly ionized enol derivatives. In ethanolic solutions, they all formed coloured complexes with ferric chloride. Non-inhibitory phenylbutazone analogues such as the 3-O-methyl enol-ether and the dicarboxy derivatives, the acyclic analogue (2-*n*-butyl-malonyldianilide, HP 342) and phenylpyrazole had no acid-quenchable light absorption at 255–265 mμ and did not form coloured complexes with ferric ions in ethanolic solutions.

Non-inhibitory pyrazolones, e.g. antipyrine, exhibited acid-quenchable light absorption at 265 mμ with concomitant increase in absorption at 236 mμ (conjugated ketone) at pH 2. Ferric chloride precipitated antipyrine and related di-N-substituted pyrazolones from ethanolic solutions. Pyrazolones which inhibited S_i metabolism, e.g. 1-Phenyl-3-methyl-pyrazole-5-one = nor-(N-demethyl)antipyrine, exhibited similar spectral properties but formed highly coloured soluble complexes with ferric chloride in ethanol.

Cinchophene gave a coloured complex with ferric ions in ethanol. Isonicotinic acid did not give a coloured complex with ferric ions in organic media. All the active salicylate analogues, tested *in vitro*, gave vivid coloured complexes with ferric ions in ethanol and in water. Inactive analogues, e.g. *m*- and *p*-hydroxy isomers, did not.

DISCUSSION

Concerning the techniques

Many investigators have tacitly assumed that ^{35}S incorporation into connective tissues signifies mucopolysaccharide biosynthesis *de novo*. This is not necessarily so, for Suzuki and Strominger[16] have shown that certain enzymes will transfer $^{35}S_i$ to preformed fully sulphated polysaccharides, e.g. chondroitin sulphates A and C. In the present experiments, it was established that glucose-^{14}C, acetate-^{14}C and $^{35}S_i$ were incorporated into the acidic polysaccharide fractions, isolated from papain digests of the incubated cartilage slices. A comparison of Table 1 with Tables 2, 3 and 4* shows (a) that individual anti-inflammatory drugs depressed the incorporation of ^{14}C *and* ^{35}S to approximately the same extent; (b) that other compounds, e.g. antipyrine, were equally inactive towards the incorporation of both ^{14}C and ^{35}S; and (c) that preincubation with glutamine stimulated both processes (^{35}S incorporation in the presence of 10^{-4} M glutamine was 145 per cent control; cf. Table 1). It seems reasonable to suppose therefore that measurements of ^{35}S incorporation into the acidic polysaccharide fractions reported here, do provide an index of either *de novo* mucopolysaccharide biosynthesis, or at least of the renewal (and extension?) of both the skeleton and the substituent groups of pre-formed mucopolysaccharides.

These observations of drug action upon polysaccharide biosynthesis and tissue respiration *in vitro* confirm and considerably extend conventional studies of the (local) action of anti-inflammatory drugs *in vivo*. Such studies commonly measure the growth of connective tissue (granuloma) around, and into, cotton pellets or plastic sponges implanted subcutaneously into small experimental animals. Drugs which will inhibit the growth of new connective tissue, e.g. phenylbutazone, hydrocortisone, have been shown to diminish sulphate incorporation into these granulomae.[17] The present experiments show that such "anti-inflammatory" (more strictly "anti-granuloma growth") drugs also inhibit biosynthetic processes in surviving tissue slices containing a high proportion of fibroblasts—the cells which synthesize the characteristic biopolymers of connective tissue. Several advantages accrue on this dispensing with the whole animal for studying drug action upon connective tissues.

Considerable reservations must be made, however, about these *in vitro* studies because:

(a) These techniques (and those of the granuloma assay) can only detect anti-anabolic drugs which act on connective tissue. Such drug actions may not be synonymous with true anti-inflammatory activity.

(b) Compounds are found to be pharmacologically active, which have little or no systemic action *in vivo*. Examples of this are known too from granuloma assays and are discussed below.

(c) Drug action is studied in the absence of inflammatory stress and in the virtual absence of normal regulators of tissue metabolism and activity, such as endogenous hormones and a physiologically controlled supply of nutrients.

Cattle cartilage and cornea have been used as representative connective tissues throughout this study, since they are readily obtained relatively free of plasma and

* Table 1, as it stands, is not strictly comparable with the other tables. Subtracting the radioactivity of PS-^{14}C (recorded as % of control) from 100 per cent, gives the degree of drug inhibition, directly comparable with data in Tables 2 to 4.

extraneous material, including blood vessels and nerves. Slices may be prepared from these two tissues in quantity which do not vary very much in individual biochemical activity and between similar experiments. Cartilage does not normally exhibit the inflammatory response of other mesenchymal/connective tissues to injury and noxious stimuli. So on theoretical grounds, it may not be an appropriate tissue with which to study the action of anti-inflammatory drugs. All experimental findings with cartilage were wholly confirmed by studies upon cornea—a tissue which is commonly inflamed and therapeutically amenable to topical applications of anti-inflammatory steroids. The polysaccharide sulphate content of cornea is much less and of different composition than that in cartilage.[5] Cornea and cartilage more nearly approximate in cellular and chemical composition to tissues which suffer inflammation than does the experimental material favoured by many other workers for drug studies *in vitro*, employing parenchymal tissues (liver, brain), yeast, other micro-organisms or considerably modified mesenchymal cells maintained in tissue culture.

Intact corneas contain a mixed population of cells. The fibroblasts of the corneal stroma lie between the surface layers of highly active epithelial and endothelial cells. Chemical analysis of horizontally sliced, and scraped, beef corneas largely stripped of epithelium and endothelium and of the scrapings, showed that the corneal mucopolysaccharides were almost entirely confined to the stroma.* $^{35}S_i$ was not significantly incorporated into *high molecular weight*-material by this epithelial fraction. $^{35}S_i$ utilization for polysaccharide sulphation by slices of whole cornea therefore represents an activity of the fibroblasts in the corneal stroma. The high metabolic activity of the epithelial cells (not entirely removed by scraping) precluded studies of drug action on the stromal fibroblasts with ^{14}C-labelled substrates.

Some biochemical properties of anti-inflammatory drugs

The experiments with $^{35}S_i$ and glucose-^{14}C have established that all four classes of anti-inflammatory drug together with cinchophene inhibit the biosynthesis of the mucopolysaccharide sulphates in cornea and cartilage. Salicylate, phenylbutazone and chloroquine (but not hydrocortisone) also inhibit mucopolysaccharide biosynthesis by beef heart valves *in vitro* (H. Boström, A. Moretti and M. W. Whitehouse, unpublished work). Inhibition of this biosynthetic sequence is probably of little therapeutic value. Nonetheless, it is a very useful (and quantifiable) index that the drugs are indeed profoundly influencing connective tissue metabolism.

It is especially significant that glucose and acetate incorporation into the polysaccharide sulphates was inhibited by the drugs. It has been argued[18] that since many anti-inflammatory drugs can be sulphated *in vivo* or by liver preparations *in vitro* (e.g. hydrocortisone,[19] salicylate,[20] phenylbutazone,[21] hydroxychloroquine[18]), the inhibition of sulphate incorporation into mucopolysaccharides is due to competition by these drugs for "active sulphate". Some drugs certainly inhibit sulphate incorporation but neither they nor their known metabolites would seem to be able to form sulphate esters *in vivo*, e.g. chloroquine.[22] Any theory presenting anti-inflammatory drugs as sulphate acceptors requires that all these drugs should be sulphated by the connective tissues, for which no evidence has yet been obtained, e.g. steroids.[23] Such a theory will

* Scraped and sliced corneal tissue was kindly provided by Dr. A. Pirie, Nuffield Department of Ophthalmology, University of Oxford.

certainly not explain the drug action upon the incorporation of glucose into the polysaccharide chain—a process which appears to proceed independently of polysaccharide sulphation.[16, 24, 25]

Cinchophene, chloroquine and phenylbutazone each partially "uncoupled" the uptake of $^{35}S_i$ by the tissues from its incorporation into the polysaccharides, causing an increase in the *proportion* of $^{35}S_i$ to total ^{35}S within the tissue. This phenomenon, previously discerned with salicylate and hydrocortisone[5] is a further indication that the drugs inhibit the utilization of ^{35}S by the cell. Tissues incubated with low levels of chloroquine and hydroxy-chloroquine ($\leqslant 10^{-3}$ M) contained much more $^{35}S_i$, liberated only by papain digestion and not by washing with saline, than the drug-free controls. But with all the other anti-inflammatory drugs and with higher concentrations of chloroquine, the quantity of $^{35}S_i$ in the drug-free incubated tissues was always less than that in tissues incubated without drugs—even though the *proportion* of $^{35}S_i$/total ^{35}S had been increased by the drug action. This suggests that all the drugs inhibit the uptake of $^{35}S_i$ from the medium by the tissues to some degree, as well as inhibiting the intracellular utilization of the $^{35}S_i$. A dual action is very clearly indicated in the case of chloroquine by the results of short term incubation experiments. To explain such a dual action it has been suggested[5] that anti-inflammatory drugs act primarily on processes generating adenosine-5′-triphosphate (ATP) within the tissues.

Some of the drugs, e.g. cinchophene and steroids, directly inhibit the oxidation of glucose and fatty acids (Table 1) so diminishing the supply of ATP derived from cellular oxidation processes coupled to phosphorylation. Other drugs could act as uncouplers of oxidative phosphorylation in the connective tissues. 2:4-Dinitrophenol (5×10^{-5} M) was found to increase the yield of $^{14}CO_2$ (170–300 per cent) from the oxidation of pyruvate-^{14}C and octanoate-^{14}C by cartilage slices, in accord with its known property of abolishing respiratory control by uncoupling the phosphorylation of adenine nucleotides from mitochondrial oxidation. This observation supports the demonstration of oxidative phosphorylation in cartilage[26] and explains the action of dinitrophenol upon mucopolysaccharide biosynthesis.[5] The data in Table 1 suggests that salicylate and phenylbutazone may each stimulate the oxidation of certain substrates, perhaps by uncoupling oxidative phosphorylation. Both these drugs, at these concentrations, will uncouple oxidative phosphorylation in rat liver mitochondria.[27, 28] The inhibition of octanoate oxidation by phenylbutazone, not observed with antipyrine and dicarboxyphenylbutazone, indicates that this particular drug may inhibit ATP generation by more than one action upon cellular oxidation.

Lindner has reported[29] that administration of phenylbutazone to rats liberates mucopolysaccharides from granulomae and causes an increased excretion of aminosugars in the urine. Other workers[30] have described the loss of unidentified sulphates from dog costal cartilage slices incubated at 37 °C, amounting to 20 per cent of the initial sulphate content of the tissue, during a 12-hr incubation. In our experiments it was found that the amount of polysaccharide sulphates shed into the incubation medium during 6-hr incubation at 37 °C was no more than 7 per cent of the polysaccharide sulphate remaining in the tissue. The presence in the medium of phenylbutazone, or of other representative anti-inflammatory drugs, did not increase the quantity of mucopolysaccharide passing into solution. No evidence was found for any drug-induced desulphation of the cartilage polysaccharides. Lindner's conclusions were based primarily on histochemical observations which probably would not

distinguish between impaired biosynthesis and increased breakdown of the tissue polysaccharides, assuming these to be in a state of continuous "turnover".

Bollet[31] has described the inhibitory effect of salicylate, phenylbutazone and other anti-inflammatory drugs upon the biosynthesis of glucosamine-6-phosphate from fructose-6-phosphate and glutamine by connective tissue derived from plastic sponge implants. Glucosamine-6-phosphate is an essential intermediate for the biosynthesis of keratosulphate and the chondroitin sulphates,[32] which are the principal sulphated polysaccharides of connective tissue. These drugs had little or no effect upon glucosamine biosynthesis in rat liver[31] and in human colonic mucosa (C. A. Pasternak and M. W. Whitehouse, unpublished experiments using described methods[33]). Anti-inflammatory drugs inhibit glutamine formation from succinate in rat liver preparations.[34] The incorporation of both glucose-^{14}C and $^{35}S_i$ into cartilage mucopolysaccharides is very sensitive to the supply of glutamine and is inhibited by glutamine antagonists such as DON. Thus, some of these anti-inflammatory drugs may depress polysaccharide synthesis by inhibiting the synthesis and utilization of glutamine and hence amino-sugar and mucopolysaccharide biosynthesis, in addition to inhibiting the generations of ATP and all dependent endergonic reactions.

The summary of our experimental findings given in Table 8 indicates that there are distinctive features in the action of the various drugs. Therefore it is highly improbable that they all act by the same mechanism. The concentration of drug for *in vitro* activity is rather similar to the plasma levels *in vivo* (where known), required to elicit anti-rheumatic activity.

TABLE 8. SOME DISTINCTIVE PROPERTIES OF INDIVIDUAL ANTI-INFLAMMATORY DRUGS

Drug	Concentration required *in vitro* ($\times 10^{-3}$M)	Reversibility of action	Immediacy of action	Inhibition of glucose oxidation	Complex formation with Fe^{3+}
Salicylate	1–5	+	+	—	+
Phenylbutazone	0·2–1·0	+	+	—	+
Hydrocortisone	0·1–0·5	+	—	+	—
Cortisone	0·5	+	(+)	+	—
Chloroquine	0·5–2·0	—	—	+	?
Cinchophene	0·5–1·0	+	+	+	+

The delayed action of hydrocortisone upon cartilage metabolism *in vitro* parallels the well known delayed physiological[35] and pharmacological[36] response to this hormone. Is it mere coincidence that chlorquine and hydroxychloroquine should both have a delayed action *in vitro* and a slow cumulative action *in vivo*? These two drugs must be continuously administered for from 1 to 3 months before any beneficial response is obtained.[37, 38] Anti-inflammatory steroids[39] and chloroquine[37] may each induce corneal lesions *in vivo*. As demonstrated, they are moderately potent inhibitors of corneal metabolism *in vitro*.

Relationship of activity to structure

When chemical analogues of the various drugs were tested for their activity upon sulphate metabolism in cornea and cartilage, it was found that their potency could be

correlated with at least two physical properties:

(a) ability to form complexes with metal ions;

(b) lipophilic character, favouring partitions into lipid rather than aqueous phases.

Salicylic acid, salicylaldehyds[40] and a number of glucocorticoids[41] can chelate cuprous and ferrous ions. Some evidence has now been obtained that phenylbutazone and its potent analogues can also form metal complexes. Wiesel[41] has shown that oxine (8-hydroxyquinoline), a powerful chelating agent, possesses anti-inflammatory properties. He reported that water soluble chelating agents were ineffective in suppressing inflammation; those compounds which are only sparingly soluble or water-insoluble being more effective in producing these anti-inflammatory effects.

The strong inhibitory activity of some sulphydryl compounds deserves comment, in view of their ability to chelate metals. In current practice gold "salts" are administered as complexes with thiols. The therapeutic activity of such gold preparations as anti-rheumatic drugs, may perhaps be associated with the slow release of the parent thiol.

Analogues of salicylate and thyroxine

The fact that acetyl salicylate, 2-methoxybenzoate, 3- and 4-hydroxybenzoates[8] had no effect on cartilage and corneal metabolism, establishes the requirement for an *ortho* phenolic group to mimic the action of salicylate *in vitro*. The inhibitory activity manifested by salicylaldehyde, salicylaldoxime and certain N-substituted derivatives of salicylaimde, indicates that the carboxyl group of salicylic acid is replaceable by other *ortho* carbonyl functions. All these compounds might be metabolized to salicylate within the tissue, though no evidence for such metabolism was found in these experiments. The further observation that *ortho* ketones (2-hydroxy- and 2:4-dihydroxy-acetophenone) were not active inhibitors suggests that the inhibitory activity of salicylate analogues is not associated with the carbonyl group *per se*. Striking increases in potency over salicylate were observed on (a) replacing the benzene ring with the naphthalene nucleus, and (b) substituting the aldehyde function for the carboxylate group. These individual increases in potency were apparently additive, for *o*-hydroxy-naphthaldehydes were even more effective inhibitors than either *o*-hydroxybenzaldehydes or *o*-hydroxynaphthoates. This suggests that less hydrophilic compounds containing the *o*-hydroxycarbonyl or *o*-hydroxycarboxyl function might be therapeutically stronger drugs than salicylate for the management of connective tissue diseases. Some further support for this hypothesis is to be found in the decline in potency with increasing hydrophilic character, in the series: *o*-cresotate, salicylate, pyrocatechuate, other dihydroxybenzoates and 5-sulphosalicylate, and in the further series: *o*-vanillin, salicaldehyde, 2:4- or 2:5-dihydroxybenzaldehydes, 2:4:6-trihydroxybenzaldehyde and pyridoxal. The dihydroxybenzaldehydes do not inhibit cellular oxidation and so may be more truly selective in their drug action than salicaldehyde.

The greater potency of L-tri-iodothyronine, and the lower potency of D-thyroxine compared with that of L-thyroxine, as an inhibitor of polysaccharide sulphation, parallels the activity of these three thyrophenols upon rat liver preparations as uncouplers of oxidative phosphorylation[42, 43] and inhibitors of cellular oxidation.[44]

Steroids

These experiments have shown that there is no simple relationship between the known anti-inflammatory property of a steroid and its effect upon corneal and cartilage biosynthesis. Anti-inflammatory analogues of hydrocortisone were potent inhibitors of polysaccharide biosynthesis *in vitro*. Progesterone, DOC (cortexone) and compound "S" were also found to be potent inhibitors. These steroids are not normally considered to be anti-inflammatory agents. However, it has been shown[36, 45, 46] that these hormones will exhibit anti-inflammatory activity if administered *locally* to granulomae even though they have little activity when administered systemically. The presence of the 11- and 21-hydroxy groups in a C_{21} steroid is apparently not an absolute prerequisite for anti-inflammatory activity.

The *in vitro* activity of DOC, compound "S" and hydrocortisone upon cartilage and cornea metabolism was not modified when these hormones were incubated with the tissue slices in the presence of beef serum. Thus the observed differences in anti-inflammatory activity between DOC and hydrocortisone *in vivo* cannot be associated merely with firmer binding of DOC than of hydrocortisone to the serum proteins. The relative impotency of progesterone, DOC and compound "S" *in vivo* must be a consequence of other processes in the whole animal which metabolize these steroids—or otherwise render them inaccessible to the inflamed tissue.

DOC, progesterone and compound "S" are potent inhibitors of cellular oxidation (and phosphorylation) not only in parenchymal tissues[5, 47] but also in mesenchymal cells, as the present experiments with cartilage slices have shown. By so inhibiting energy-yielding catabolic processes within the cell, these compounds would also inhibit endergonic reactions, such as polysaccharide biosynthesis. They might also be expected to severely modify endergonic processes involved in the inflammatory response such as water movement, tissue swelling, general cellular reactivity, etc.

The only steroids known to be anti-inflammatory *in vivo* but which did not inhibit cartilage and corneal metabolism, were highly polar derivatives (phosphate, sulphate, hemisuccinate ester) which presumably failed to penetrate into the tissues, and prednisone.

Cortisone appeared to have a different action upon cartilage than hydrocortisone, distinguished by a more rapid but less-sustained inhibitory effect upon polysaccharide biosynthesis. These findings confirm that hydrocortisone is indeed a more potent anti-inflammatory agent than cortisone, as indicated by other studies.[36, 48] The relative lack of a lag period in cortisone action *in vitro* suggests that cortisone may, however, penetrate into the tissues more rapidly than hydrocortisone.

Phenylbutazone and analogues

Amongst these compounds good agreement has been found between therapeutic efficiency as an anti-rheumatic drug and potency as an inhibitor of cartilage and corneal metabolism *in vitro*. The enol form of phenylbutazone appears to be the physiologically active form of the drug. The methyl enol-ether is devoid of activity *in vivo*,[11] and *in vitro*. Hence, phenylbutazone contains a conjugated α-keto-enol system, viz. $CO—\underset{|}{C}=\underset{|}{C}—OH$, also present in salicylates.* Table 8 indicates a range of properties shared by salicylates and phenylbutazone. At a concentration of 10 mg % (ca. 3×10^{-4} M), phenylbutazone stimulates the oxidation of pyruvate, succinate,

*iso*citrate, fumarate and malate by rat liver mitochondria,[49] supporting the concept that (like salicylate) the drug is an uncoupler of oxidative phosphorylation. At higher concentrations, phenylbutazone inhibits the oxidation of these substrates and especially of α-oxoglutarate. This may explain why octanoate oxidation by cartilage slices is so very sensitive to the drug at 10^{-3} M. Schellenberg[50] has drawn attention to the general toxic action of phenylbutazone, sulphinpyrazone and their metabolites at concentrations greater than 3×10^{-4} M upon cells in tissue culture. The relationship between lipid solubility and activity *in vitro* is very pronounced amongst phenylbutazone analogues. Increasing the water solubility seems to depress the drug potency, as reflected in the series: phenylbutazone, 4′-monocarboxy-phenylbutazone, 4:4′-dicarboxy-phenylbutazone.

Antipyrine, consistently without effect on cartilage and corneal metabolism, does not uncouple oxidative phosphorylation in rat liver mitochondria at 2×10^{-3} M.[27] The finding of *in vitro* "anti-sulphation" activity amongst phenyl-pyrazolones with an unsubstituted imino group (N_2), suggests that some of the newer monoamine oxidase inhibitors ("psychic energizer") which are pyrazolones based on phenylhydrazine, will possess some anti-inflammatory activity. The effect of lipid solubility upon activity is again demonstrated, amongst pyrazolones, by the relative potencies of (in declining order): 1,3-diphenyl-pyrazol-5-one, picrolonic acid, 1-phenyl-3-methyl-pyrazol-5-one, 1-phenyl-pyrazol-5-one-3-carboxylate and antipyrine (inactive).

Chloroquine, cinchophene, etc.

The anti-rheumatic properties of an anti-malarial drug were first described for mepacrine (quinacrine).[5] This compound is moderately firmly bound by the tissue mucopolysaccharides. Greiling[52] has described the similar binding of chloroquine (Resochin). The principal action of these drugs may therefore be one of immobilizing the tissue polysaccharides, effectively poisoning their physiological function. Other organic bases such as Rivanol, quinine and promethazine likewise inhibited polysaccharide sulphation and were bound to the tissues. Unlike the antimalarials, they did not exhibit any selective action in "uncoupling" S_i uptake by the tissues from its subsequent utilization for polysaccharide sulphation. These organic bases were also potent inhibitors of cartilage respiration.

The relationship of drug potency to lipophilic character is manifested again in the series (in order of increasing potency): hydroxychloroquine, chloroquine, mepacrine, santoquin.

The pharmacological properties of 8-hydroxyquinoline (oxine) as a fungicide and bactericide[53] and as a rather novel anti-inflammatory agent[41] have been correlated with its metal-chelating properties. These properties seem to determine its powerful action upon polysaccharide sulphation *in vitro*. Its isomer 2-hydroxyquinoline is almost devoid of both *in vitro* activity and ability to form metal chelates.

Cinchophene provides another example of the apparent relationship between lipid solubility and activity as a drug *in vitro*. The "stripped-down" analogue, isonicotinic acid, is virtually inactive whilst quinoline-4-carboxylic (cinchoninic) acids exhibit some activity but are much less potent than cinchophene itself. Cinchophene is an ulcerogenic agent.[54] Gastric ulceration is a common side reaction attending the administration of many conventional anti-inflammatory drugs. Perhaps these drugs,

and cinchophene, depress the maintenance and biosynthetic activities of the gastric mesenchymal cells.

Acknowledgements—We thank Professor G. Biörck for his interest in this work. It is also a pleasure to acknowledge the assistance of the Misses T. Bennet and A. Jarvis and Messrs. K. Berntsen and H. Johnsson. We are indebted to Dr. S. Ullberg (Royal Veterinary College, Stockholm) for the use of a liquid scintillation counter and to Dr. D. S. Parsons (Oxford) for the loan of a tissue slicer.

Various drugs were generously donated by Dr. C. A. Pasternak (Oxford), Dr. D. Kritchevsky (Philadelphia), Imperial Chemical Industries Ltd., Geigy Pharmaceutical Co., Parke, Davis and Co., Smith and Nephew Research Ltd., May & Baker Ltd., Merck, Sharp and Dohme, Schering Corporation, Beyer Products, and Cyanamid of Gt. Britain.

This investigation was supported by grants to the University of Oxford from the Rockefeller Foundation, New York, N.Y., and the National Institutes of Health, Bethesda, Maryland, U.S.A.; to the Wenner-Gren Institute, University of Stockholm, from the National Institutes of Health (A-2568) and King Gustaf the Fifth's Eightieth Birthday Fund, and the award of a Research Fellowship to M. W. W. by Exeter College, Oxford.

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