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BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—VII.

INHIBITION OF PROTEOLYTIC ENZYMES IN CONNECTIVE TISSUE BY CHLOROQUINE (RESOCHIN) AND RELATED ANTIMALARIAL/ANTIRHEUMATIC DRUGS

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Abstract—A chondromucoprotease present in bovine cartilage, with optimal activity at pH 5, has been partially characterized. This autolytic enzyme was irreversibly inhibited by chloroquine, those of its metabolites with a quinoline nucleus, and mepacrine (quinacrine, atebrine). These drugs did not affect cartilage degradation by a mucopolysaccharase (ovine hyaluronidase) or papain.

At relatively high concentrations (10 mM), chloroquine and some of its metabolites inhibited rat skin collagenase, a presumed collagenase in bovine cartilage and a bacterial collagenase (clostridiopeptidase A). This drug action was reversible and non-competitive with the substrate, when this was gelatine. Rivanol and cetyl ammonium salts (0·25 mM) were powerful inhibitors of clostridiopeptidase A.

Other types of anti-inflammatory (antirheumatic) drugs did not inhibit either the chondromucoprotease or the collagenases. A neutral sulphydryl-dependent esterase in bovine nasal cartilage was inhibited by high levels of chloroquine.

Chloroquine and hydroxychloroquine were metabolised (de-ethylated) by bovine cartilage incubated at pH 7·4.

The possible value of (a) chloroquine and mepacrine for treating degenerative arthritis, and of (b) cetyl ammonium salts for treating clostridial infections, is discussed.

DURING an earlier study of the biochemical properties of chloroquine and related antimalarials with antirheumatic activity, it was noted that the proteolytic digestion of cartilage slices by an exogenous plant protease (papain) proceeded less readily if these tissue slices had been preincubated with the antimalarials.[1] Glick and his colleagues[2] had previously reported that the enzymic hydrolysis of hyaluronate by a mammalian mucopolysaccharase (testicular hyaluronidase) was inhibited by certain antimalarials which were 8-aminoquinoline derivatives. These two observations suggested that perhaps chloroquine (resochin, Aralen) and other antimalarials might protect macromolecular components of connective tissue from degradation by hydrolytic enzymes (proteases, mucopolysaccharases), released either from infiltrating leukocytes or from within the tissues (lysosomes), as a consequence of inflammation or rheumatogenic stimulus.

It subsequently proved that neither of these assumptions was valid. It was found that chloroquine fails to inhibit the action of both papain and hyaluronidase *in vitro*. However, we found that chloroquine and related antimalarials inhibited at least two endogenous proteolytic enzymes, present in skin and cartilage, as well as an

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exogenous collagenase of bacterial origin (clostridiopeptidase A). An attempt was made to locate the site(s) of drug action and delineate the chemical features essential for drug activity. Cartilage preparations were found to metabolise chloroquine and hydroxychloroquine. Some of the metabolites were tentatively identified and also tested for their protective (anti-protease) activity.

A preliminary account of this work has been given.[3]

MATERIALS

The source of many drugs has been detailed.[4] Other drugs and reagents were obtained from the following sources:

Benger's Laboratories, Holmes Chapel; ovine testicular hyaluronidase B.P. ('Hyalase').

British Drug Houses Ltd., Poole; crystalline papain and trypsin, soybean trypsin inhibitor, picryl (2,4,6-trinitrobenzene) sulphonic acid, N-ethylmaleimide, toluene-α-fluoride.

Koch–Light, Colnbrook; diazotised collagen (Azocoll).

Kodak, Liverpool; thionine, all *trans* vitamin A alcohol and acid (Retinol, Retinoic acid).

Charles Lennig & Co., London, W.C.1.; Triton X-100.

May & Baker, Dagenham; primaquine phosphate, 4-amino-7-chloroquinoline, 5-diethylamine-2-aminopentane and 5-diethylaminopent-2-one,.

New England Nuclear Corp., Boston, Mass.; (ring)-3-^{14}C-chloroquine.

Sigma Chemical Co., London, S.W.6; collagen (from tendon), collagenase (from *Cl. histolyticum*).

Sterling-Winthrop Research Institute, Rensselaer, N.Y.; desethylchloroquine (SN-13616, Win 10022), didesethylchloroquine (SN-13617, Win 10751).

Chondroitin sulphates (mixtures of A and C) were extracted from cartilage[5] or obtained commercially (Sigma). Hyaluronate was obtained from *Streptococcus.* Chondromucoprotein was prepared from beef nasal cartilage by a procedure combining features of both the A and B methods of Malawista and Schubert[6] using a Virtis high-speed tissue homogenizer. Bovine cartilage was obtained from the local slaughterhouse within 2 hr of slaughter, then kept at 2° and used within the next 2 hr. Cartilage slices were cut mechanically or freehand to a thickness of approx. 0·5 mm and kept in chilled isotonic saline until used. Rabbit ear cartilage was freed of skin and cut into narrow strips.

EXPERIMENTAL METHODS

Hyaluronidase action on soluble substrates, i.e. hyaluronate, chondroitin sulphates (CS) and chondromucoprotein (CMP), in sodium acetate buffers (0·1 M) was followed by reduction in the turbidities produced with Rivanol (Ethodin) in ammonium formate solutions (0·8 M formate for CS and CMP, 0·1 M for hyaluronate determinations)[5, 7]. Cartilage degradation by hyaluronidase (100 IU/ml) was followed by release of oligoglucuronides into solution, determined colorimetrically with orcinol[7] or carbazole.[8]

Papain digestion of soluble substrates (2 mg/ml casein, plasma albumen, chondromucoprotein) in 0·1 M sodium phosphate buffer pH 6·8 at 37° was followed by determining phenolic peptides soluble in 0·3 N trichloracetic acid, or in Rivanol solutions

at pH 1 (with CMP digests only), using the Folin and Ciocalteu phenol reagent.[9] As cysteine interferes with this determination, the papain was activated[5] by pre-incubation with 10 mM sodium cyanide (i.e. not with cysteine). Digestion of dried nasal cartilage (10 mg) with crystalline papain (0·3 mg per incubation) or trypsin (50 μg) was followed by measuring the acidic polysaccharides solubilized, using the Rivanol or orcinol reagents (see above).

The activity of an endogenous chondromucoprotease in bovine nasal, bovine tracheal and rabbit ear cartilages was assayed by determining acidic polysaccharides released at pH 5·0 and 37° on incubating these tissues for periods up to 24 hr.

Bacterial collagenase was determined by the formation of soluble peptides, or increase in aminogroups, on incubating the commercial enzyme (300 μg/ml) with cartilage slices (20 mg dry weight), insoluble collagen (20 mg), "azocoll" (5 mg) or gelatine (3 mg/ml) in 0·1 M sodium phosphate buffer, pH 7·4 at 37°. Aminogroups were determined with ninhydrin[10] or picrylsulphonic acid.[11] Mammalian collagenase preparations (rich in ninhydrin-reactive material) were assayed by determining the soluble hydroxyproline-containing peptides released from insoluble collagen or from cartilage slices at pH 5·0 (citrate) and at pH 7·0 or 7·5 (phosphate); hydroxyproline being determined after acid hydrolysis.[12]

Latent collagenase[13] was extracted from shaved abdominal skin of 300 g. Wistar rats. The skin was dissected with scissors from underlying tissue, cut into small pieces and frozen in liquid nitrogen, then pulverized in a chilled mortar. The lumpy powder obtained was continually stirred for 4 hr at 2° with 0·15 M sodium chloride (8 ml/wet weight skin) with occasional high-speed homogenization for periods of not more than 1 min. After centrifugation at 2° for 20 min at 4000 *g* the supernatant fluid was dialysed overnight against distilled water and reduced to one-fifth volume by lyophilization. This concentrate was briefly activated with trypsin (1 mg/12 ml concentrate) at pH 5·5, the trypsin neutralized with 4 mg soybean inhibitor and the collagenase activity assayed at pH 5·5 with insoluble collagen as the substrate, as described by Goldstein, *et al.*[12] Further extractions of the skin residue (overnight at 2°) yielded more (latent) collagenase.

Cartilage esterase was determined by the appearance of the nitrophenolate ion in solutions containing bovine nasal cartilage slices incubated at room temperature or 37° for periods up to 30 min with 3 mM *p*. nitrophenyl acetate (added last in 25μl N,N-dimethylformamide), 0·1 M sodium phosphate pH 7·4 and drugs. This enzymic activity was terminated by adding 1 ml 0·15 M sodium citrate pH 5·0 per 2 ml incubation medium.

Chloroquine metabolites were detected by thin layer chromatography,[14] on fluorescent silica (Kieselgel HF_{254}, Merck, Darmstadt).

RESULTS

The effects of chloroquine and other drugs upon the following enzyme systems was investigated.

1. *Testicular hyaluronidase on hyaluronate and chondroitin sulphates (A and C).* Chloroquine diphosphate (5·4 mM) and mepacrine hydrochloride (5·6 mM) did not effect the rate of action of this enzyme in degrading (a) hyaluronate at pH 4·5 or 6·0 and (b) chondroitin sulphate(s) at pH 6·0 in the presence of 0·15 M sodium chloride.

Glick and co-workers reported that 6-methoxy-8-β-di(isobutyl)-aminomethyl-quinoline inhibited hyaluronidase.[2] We found that another 8-aminoquinoline anti-malarial, primaquine phosphate ($\leqslant$ 35 mM) actually stimulated the digestion of chondroitin sulphate(s) by hyaluronidase at pH 6·0, probably by reversing the effect of (excess) substrate which tends to inhibit this enzyme. At similar high drug levels, chloroquine phosphate and chloroquine sulphate also apparently increased the rate of chondroitin sulphate digestion. This drug effect was not wholly due to the (small) stimulation of hyaluronidase activity observed with phosphate and sulphate ions alone (Table 1). Mepacrine hydrochloride (35 mM) had no effect on the rate of chondroitin sulphate digestion.

2. *Testicular hyaluronidase on whole cartilage slices or extracted chondromucoprotein.* Neither chloroquine or primaquine inhibited the action of this enzyme on these "more native" substrates. In fact, these drugs enhanced hyaluronidase activity in the same manner as they stimulated the digestion of chondroitin sulphate, discussed above (Table 1).

TABLE 1. EFFECTS OF CHLOROQUINE PRIMAQUINE AND INORGANIC SALTS ON HYALURONIDASE DIGESTION OF CHONDROITIN SULPHATE AND CHONDROMUCOPROTEINS

Substrate		Relative digestion in the presence of Na_2SO_4	NaH_2PO_4	CQS_i	CQP_i	PQP_i
Chondroitin sulphate	1·0	1·04	1·11	1·15	1·23	1·32
Chondromucoprotein	1·0	1·00	1·30	1·42	1·42	1·75

Digestion measured by reduction in the amount of material forming a turbidity with Rivanol. after 1½ hr incubation at pH 6·0 and 37° with the enzyme. Drugs and salts were 20 mM. Digestion, in drug-free controls = 1·0.

CQ = chloroquine, PQ = primaquine, P_i = phosphate, S_i = sulphate.

Cartilage slices were also preincubated with chloroquine (up to 50 mM) for 60 min before adding hyaluronidase: even under these conditions, there was no evident reduction in the hyaluronidase susceptibility of this tissue.

Chloroquine forms complexes with polysaccharide sulphates.[15, 16] Control experiments showed that mepacrine and primaquine formed insoluble complexes with chondroitin sulphate in solutions of very low ionic strength, more readily than chloroquine. These precipitates were all soluble in the 0·1 M dilute salt (buffer) solutions normally used for these studies with hyaluronidase. In solutions of lower ionic strength, the precipitated chondroitin sulphate or chondromucoprotein were still susceptible to enzymic digestion. These drugs did not interfere with the turbidometric determination of polysaccharides with Rivanol.

It must be concluded that these particular basic drugs do not bind sufficiently strongly to acidic mucopolysaccharides to prevent their degradation by mammalian mucopolysaccharases with properties similar to testicular hyaluronidase.

3. *Papain on soluble animal proteins and chondromucoprotein.* The rates of digestion of gelatine, plasma albumen, casein and haemoglobin by cyanide-activated papain were not affected by chloroquine phosphate or mepacrine hydrochloride (4 mM). The digestion of bovine nasal chondromucoprotein, liberating Rivanol-soluble peptides was insensitive to 16 mM chloroquine.

4. *Papain and trypsin on cartilage slices.* Oven-dried (at 120°) or freeze-dried nasal and tracheal cartilage slices were digested at pH 6·8 and pH 4·0 (with papain) and pH 7·4 (with trypsin) with varying proportions of enzyme to tissue. Chloroquine phosphate (10 mM) had no effect on the rate or extent of tissue digestion, even when the slices were preincubated with the drug before treatment with these proteases.

5. *Bacterial collagenase on insoluble collagen and gelatine.* Chloroquine salts partially inhibited the actions of clostridiopeptidase A from *Cl. histolyticum* upon these two substrates. A given concentration of the drug inhibited degradation of both substrates, by a given amount of enzyme, to almost exactly the same extent (e.g. approx. 50 per cent with 5 mM drug)—suggesting that the drug combined with the enzyme rather than with the substrates. This was confirmed by further experiments using gelatine as substrate: the degree of digestion varied with the amount of enzyme and drug present but was virtually independent of the gelatine concentrations. A "double reciprocal" plot (i.e. 1/V against 1/S), of data obtained in experiments with varied gelatine concentration(s), constant amount of enzyme and three different concentrations of chloroquine, confirmed that the inhibition of gelatinase activity was non-competitive with respect to substrate. K_m determined graphically was between 2 and 4 mg gelatine/ml and K_i for chloroquine was 7 ± 2 mM. The specificity of this drug action was also indicated by the fact that the digestion of both gelatine and diazotised collagen ("azocoll") by crystalline papain and trypsin, was almost insensitive to 15 mM chloroquine.

Chloroquine inhibition of this bacterial enzyme was apparently reversible. When the enzyme and drug (15 mM) were preincubated for 30 min at 37°, then diluted to give drug concentrations $\leqslant$ 5 mM before adding the substrate; the rate of collagen solubilization was almost identical with that in parallel incubations with the same (low) drug levels, and certainly faster than in the presence of 15 mM chloroquine.

Mepacrine and primaquine also inhibited this bacterial collagenase. Some potential metabolites of chloroquine[4, 14] were also examined for this property. Desethylchloroquine was approximately equipotent with primaquine and chloroquine: these were all slightly less effective at 10 mM than mepacrine *in vitro*. 4-Amino-7-chloroquinoline was less potent than desethylchloroquine. Compounds not affecting the enzyme activity when added at 20 mM included sulphydryl reagents (iodoacetate, N-ethylmaleimide), other antirheumatic drugs (amidopyrine, gold sodium thiomalate and gold sodium thiosulphate, sodium salicylate), 6-aminohexoic acid, and fragments of the chloroquine molecule representing possible metabolites derived from the aliphatic side chain of this drug, e.g. 5-diethylamino-2-aminopentane, 5-diethylamino-pent-2-one. Certain aliphatic or heterocyclic amines were notably more potent than these antimalarials in antagonising clostriopeptidase digestion of azocoll. Rivanol, cetylpyridinium bromide and cetyltrimethylammonium chloride reduced the rate of "azocoll" digestion by more than 50 per cent when present at 0·25 mM.

6. *Bacterial collagenase on cartilage slices.* Slices of dried nasal cartilage were heated at 120° to inactivate the autolytic enzyme (see later, Section 8) and then treated with clostridiopeptidase. Digestion was followed by measuring the release of (a) mucopolysaccharides, (b) ninhydrin-reactive material and (c) hydroxyproline peptides. Solubilization of all these materials was greater at pH 7·0 than at pH 5·0. Chloroquine (20 mM) stimulated polysaccharide release by this enzyme preparation but reduced peptide liberation by more than 50 per cent.

7. *Endogenous collagenases of bovine nasal cartilage and rat skin.* Incubating cartilage-slices alone at 37° liberated into solution, small but significant amounts of hydroxyproline-containing peptides, of the order of 1μg hydroxyproline/mg cartilage dry weight/20 hr at pH 5·0 and rather less than this at pH 7. This solubilization process was inhibited approximately 50 per cent on incubating the slices with 20 mM chloroquine phosphate. The presence of a potent bacteriostat (0·25 mM thiomersal), in the medium hardly affected this presumed "collagenase" activity at pH 5.0, suggesting that the enzyme was not derived from contaminating microorganisms. Hydroxyproline release from incubated cartilage slices predried at 120° was actually greater (at least twofold), than that from undried tissues but was still chloroquine-sensitive.

A latent collagenase was extracted from the abdominal skin of male Wistar rats and activated with trypsin as described by Goldstein and co-authors.[13] The weak collagenase activity obtained was inhibited more than 70 per cent in the presence of 20 mM chloroquine. This drug was only added after trypsin activation and so the chloroquine must have inhibited this mammalian collagenase—not the trypsin itself (which either hydrolyses a collagenase zymogen or destroys an endogenous collagenase inhibitor).

8. *An endogenous cartilage protease.* In the course of control experiments (for 2 and 4 above), it was observed that some degradation of bovine tracheal and nasal slices occurred when they were incubated without added enzymes in acetate buffers pH 6·0 at 37°; indicated by the appearance of mucopolysaccharide material in solution. This "solubilization" process was retarded by chloroquine and was subsequently traced to the action of a chloroquine-sensitive endogenous (autolytic) protease, which could be characterized as follows:

(*a*) The pH optimum lay between 4·5 and 5·0 in citrate-phosphate buffers, and the enzyme activity declined rather sharply with change of pH, e.g. being approx. 50 per cent that at the pH optimum at 3·8 or 6·5.

(*b*) It was either moderately soluble or moderately labile. Pre-incubating cartilage slices in unbuffered saline solutions, in Krebs-Ringer medium (pH 7·4) or in acetone at 37° for 3 hr, before determining the subsequent polysaccharide release at pH 5, largely destroyed this autolytic activity. This was not due to loss of "substrate": exogenous proteases (e.g. papain) were still able to release considerable amounts of polysaccharide from slices depleted of the autolytic enzyme, by these pre-incubation procedures.

(*c*) The temperature sensitivity of this enzyme resembled that of many hydrolases. Activity, measured at 37°, was retained by tissues frozen at −15° for several days or immersed in liquid air for 30 min but was destroyed by drying the slices to constant weight at 120°. Tissues oven-dried at 120° for only a few minutes retained some activity. Enzyme activities at 4° and 55° were respectively 7 and 120 per cent that at 37°.

(*d*) The soluble autolysis product released from cartilage by this enzyme was a chondromucopeptide, i.e. the enzyme was a protease, not a mucopolysaccharase. This autolysis product contained several aminoacids and galactosamine, and gave exactly the same ratio of glucuronate (determined with orcinol) to polyanionic material (determined with Rivanol) as was given by a soluble chondromucopeptide prepared by the action of papain on exhaustively dried (120°) cartilage slices. By contrast, the oligoglucuronide products from hyaluronidase digestion of undried

cartilage slices gave a glucuronate:polyanion(s) ratio at least 5 times that given by the chondromucopeptide(s) liberated with papain. (In this hyaluronidase digestion, the endogenous protease was largely inhibited by adding chloroquine to the incubation medium).

This autolysis product was quantitatively precipitated by Rivanol at pH 1,[6] stained metachromatically with thionine when dried on paper, and had a mobility intermediate between that of chondroitin sulphate and chondromucoprotein on paper ionophoresis in 0·2 M borate buffer, pH 8·0. The papain-liberated chondromucopeptide also exhibited these properties.

(*e*) The autolytic activity was insensitive both to (i) bacteriostatic drugs, other than antimalarials, e.g. 1·25 mM thiomersal, 100 mM iodacetate, 50 mM cyanide, 0·5 mM 2,4-dinitrophenol and 20 mM N-ethylmaleimide, and to (ii) inhibitors of chymotrypsin, trypsin or plasmin-like enzymes, e.g. 100 mM 6-aminohexoic acid, 50 mM methyl 6-aminohexoate,[17] 5 mM 2,4,5-trinitrobenzaldehyde,[18] toluene-α-sulphonyl fluoride[19] (pre-incubated with cartilage at pH 7) and nitrophenyl acetate.[20]

(*f*) A chloroquine-sensitive enzyme could be extracted in very unreproducible, and always low, yields by homogenizing cartilage in distilled water or isotonic saline at 10,000 rev/min (ice bath), which released ninhydrin-reactive material from chondromucoprotein. This particular enzymic activity was insensitive to soybean inhibitor. These active cartilage extracts did not degrade collagen or gelatine at pH 5·0 or 7·4 or hydrolyse either L-lysine methyl ester or N-acetyl L-tyrosine ethyl ester at pH 7·4.

(*g*) Only a limited number of peptide bonds were attacked by this protease, indicated by the low ratio of ninhydrin-reactive material: polysaccharide released into solution from cartilage slices at pH 5.0. Bacterial collagenase which also released polysaccharides from cartilage gave much higher yields of soluble ninhydrin-reactive material relative to polysaccharide.

(*h*) Bovine nasal cartilage appeared to be much richer in this enzyme than bovine tracheal cartiilage.

(*i*) The enzymic activity at pH 5·0 was insensitive to many conditions known to affect release of lysosomal enzymes, such as repeated freezing and thawing of the cartilage or incubation with Triton X-100 (5 mg/ml) and hydrocortisone (0·4 mM). Incubating cartilage slices with vitamin A alcohol or acid (10 μg/ml) stimulated the proteolytic activity at pH 5 by 30 per cent or less.

Mucopolysaccharide solubilization at pH 7 was approximately 5 per cent of that at pH 5 and not affected by 20 mM chloroquine phosphate or vitamin A derivatives.

Drug-sensitivity of the autolytic chondromucoprotease

The chloroquine-sensitivity of this enzyme at pH 5 varied with both the source of the cartilage and the actual autolytic activity of a given type of cartilage. 10 mM Chloroquine sulphate or phosphate inhibited the enzyme 15–85 per cent depending on the cartilage preparation used. In some instances, the enzyme activity declined continuously as the chloroquine concentration in the medium was increased: in other instances, a critical threshold concentration of chloroquine was required, below which no inhibition of the enzyme was apparent. Occasionally and with certain batches of cartilage, low concentrations of chloroquine (less than 5 mM) actually increased the quantity of polysaccharide released at pH 5 and the chloroquine con-

centration then required to inhibit the enzyme was always greater than was commonly found necessary to inhibit the enzyme in "more normal" cartilage. Bovine nasal cartilage usually released more polysaccharide than bovine tracheal cartilage and was generally less sensitive to a given concentration of chloroquine (this was not due to less overall metabolic activity in the tracheal cartilage, which actually incorporated ^{35}S-sulphate ions into polysaccharide sulphates more readily than did nasal cartilage). With batches of cartilage particularly rich in this autolytic enzyme (releasing 16–20 per cent of total tissue polysaccharides in 24 hr), 10 mM chloroquine partly inhibited autolytic digestion at 55° but higher drug concentrations (up to 25 mM) were needed to inhibit autolysis at 37° to the same degree. Collectively, these and other observations indicated that the extent to which the chondromucoprotease was inhibited in these *in vitro* experiments, depended largely on the rate of chloroquine uptake by the tissue including penetration into the chondrocytes—not just fixation by extracellular basophilic material.

This autolytic activity was always inhibited when the cartilage slices were pre-incubated with chloroquine for periods up to 6 hr at 37° and pH 7·4, then washed and transferred to a *drug-free* medium (pH 5·0) to determine the subsequent autolytic activity. Studies with ^{14}C-chloroquine established that the drug was progressively taken up by the cartilage slices even after 3 hr incubation at 37°, pH 7·4. Table 2 indicates the relative quantities of polysaccharide released into the second (drug-free) medium after pre-incubation with certain drugs closely related to chloroquine. When bovine cartilage was pre-incubated with chloroquine or mepacrine at 4° and pH 7·4, the amount of polysaccharide subsequently released into the drug-free medium at pH 5 and 37° was much greater than the polysaccharide released under the same conditions by cartilage which had been pre-incubated for the same period with the same concentration of these drugs but at 37° (the thickness of the cartilage slices was rather critical for these preincubation experiments: if they were too thin, little autolytic activity was apparent in the second incubation period).

The same relative order of drug potency indicated in Table 2, namely mepacrine > primaquine > chloroquine = desethylchloroquine, was also found in other experiments in which cartilage slices were incubated at pH 5·0 with fairly high concentrations (5–25 mM) of these drugs (i.e. no pre-incubation); the polysaccharide released into the medium being determined after at least 4 hr incubation at 37°. The following chloroquine metabolites;[4, 14] didesethylchloroquine, hydroxychloroquine, 4-amino-7-chloroquinoline (all at 10 or 20 mM) and N(7-chloro-4-quinolyl)-alanine (7·5 mM) were less effective inhibitors of the chondromucoprotease (in this order) than chloroquine at the same concentrations. Mepacrine–10, N^{ω} -dioxide (10 mM) was less effective than mepacrine. The following compounds had no effect on the autolytic activity: quinoline (50 mM), 5-diethylamino-pent-2-one and 5-diethylamino-2-aminopentane (100 mM), sodium salicylate, gold sodium thiomalate and thiosulphate, aminopyrine (all at 25 mM).

Inhibition of the autolytic enzyme by 20 mM mepacrine hydrochloride or chloroquine phosphate was not diminished by co-incubation with lysosomal activators, e.g. vitamin A (10 μg/ml), Triton X-100, bacteriostats and respiratory inhibitors (thiomersal, cyanide etc.), or previous freezing and thawing of the cartilage (to disrupt lysosomes). This last procedure actually increased the sensitivity of the chondromucoprotease to a given concentration of chloroquine.

9. *Rabbit ear chondromucoprotease.* This autolytic enzyme had a pH optimum between 4·5 and 5·0 and was 40 per cent inhibited by 10 mM chloroquine phosphate.

10. *An endogenous sulphydryl enzyme (esterase).* Fresh cartilage preparation readily hydrolysed *p.* nitrophenylacetate at pH 7·4. The yield of nitrophenol was proportional to the amount of cartilage over short incubation periods (15 min). This enzymic

TABLE 2. RELATIVE POTENCIES OF SOME ANTIMALARIAL DRUGS IN INHIBITING THE AUTOLYTIC CHONDROMUCOPROTEASE PRESENT IN BOVINE NASAL AND TRACHEAL CARTILAGE

Drug	Polysaccharide released from tracheal cartilage after preincubation period		Polysaccharide released from nasal cartilage after preincubation period	
	2 hr	6 hr	2 hr	6 hr
None	1·0	1·0	1·0	1·0
Chloroquine phosphate	0·87	0·08	0·80	0·76
Mepacrine hydrochloride	0·29	0·03	0·75	0·37
Primaquine phosphate			0·88	0·32
Desethylchloroquine	0·98	0·13	0·91	0·53
4-amino-7-chloroquinoline	0·97	0·08	1·08	0·78

Cartilage slices were pre-incubated with drugs (15 mM) for 2–6 hr at 37° and pH 7·4, washed several times with 0·15M NaCl and re-incubated in a drug-free medium. Enzyme activity measured by polysaccharide released into the drug-free medium at pH 5·0 and 37°.

activity was abolished by pre-incubation of cartilage slices with iodoacetate (1 mM) for 5 min, 50 per cent inhibited by preincubation with 20 mM chloroquine for 1 hr and inhibited to a lesser extent by 20 mM chloroquine when this drug was pre-incubated for only 5 or 10 min with the cartilage slices.

Esterase activity at pH 5·0 was very small compared with that at pH 7·4 and virtually insensitive to iodoacetate and chloroquine.

Metabolism of chloroquine and hydroxychloroquine by cartilage slices

Evidence was obtained that these two drugs were partly metabolized by bovine nasal cartilage incubated in sodium phosphate buffer, pH 7·4 at 37°.

Cartilage slices (300 mg wet weight) were incubated with ^{14}C-chloroquine (5 μc) or 20 mM non-radioactive chloroquine and hydroxychloroquine phosphates for periods from 15 min to 6 hr then washed in isotonic saline, dried and digested with cysteine-activated crude papain.[5] Each cartilage digest (2 ml) was made alkaline with sodium hydroxide and extracted twice with 0·25 ml dichloromethane. These dichloromethane extracts were applied to thin layer plates of fluorescent silica gel, and eluted with ammonia (sp. gr. 0·88)-2-propanol-ethyl acetate (5:15:85 v/v). The drugs and their metabolites were visualised by inspection under u.v. light or by autoradiography using Kodak X-ray films.

After 15 min incubation with cartilage, only chloroquine or hydroxychloroquine and traces (?) of desethylchloroquine were present in the papain digests of the cartilage: only one radioactive band corresponding to chloroquine was detected. Evidently the

tissue digestion and chromatographic procedures introduced no major artefacts ("spurious metabolites"). After 3 hr incubation, at least two u.v.-absorbing (and radioactive) zones were detected on the thin layer plates. After 6 hr incubation, these extra zones were fairly well pronounced. Their R_f values (Table 3) corresponded to

TABLE 3. PROVISIONAL IDENTIFICATION OF CHLOROQUINE AND HYDROXYCHLOROQUINE METABOLITES

Compound(s)	u.v.—absorbing zone at R_f			
Chloroquine metabolites	0 (f),	0·31 (w),	0·46 (f),	0·55 (s)
Hydroxychloroquine metabolites	0 (w),	0·19 (w),	0·32 (f)	0·44 (s)
Cartilage extracts (no drug)				0·52 (f)

R_f values for chromatography on silica gel eluted with ammonia-isopropanol-ethyl acetate. Metabolites extracted from cartilage slices incubated 6 hr at 37° with the drugs.
s = strong w = weak f = faint
R_f of reference compounds: chloroquine, 0·54; hydroxychloroquine, 0·45 desethylchloroquine, 0·33; 4-amino-7-chloroquinoline, 0·64.

(i) desethylchloroquine and hydroxychloroquine (from incubations with chloroquine) and to (ii) desethylchloroquine and a metabolite more polar than desethylchloroquine, tentatively identified as desethylhydroxychloroquine (from incubations with hydroxychloroquine). 4-Amino-7-chloroquinoline, a known metabolite of chloroquine,[14, 21] was not found in these cartilage extracts but more polar, fluorescent metabolites (quinoline acids?) were always detected. These were immobile in the solvent system used and were formed in greater yield from hydroxychloroquine than from chloroquine.

DISCUSSION

These findings indicate that basic antirheumatic drugs may, like acidic anti-inflammatory drugs,[18] specifically inhibit certain (autolytic) proteases and protect connective tissues in inflammatory conditions.

At first sight, the drug levels required to inhibit the chondromucoprotease and connective tissue collagenases may seem to be very high (10 mM or greater) and physiologically unrealistic. Nevertheless we believe these observations may have some significance in explaining the beneficial effects of these clinically slow-acting drugs in rheumatic disease. Connective tissues are able to fix these antimalarials, sometimes in considerable quantities, by virtue of their content of acidic mucopolysaccharides which may be as high as 25 per cent or more of the tissue dry weight in the case of cartilage. Furthermore, these polar drugs only penetrate cartilage tissue rather slowly and having done so, their drug action is not readily reversed by transferring the drug-impregnated tissue to a drug-free medium (see Results section and Ref. 1). Therefore the concentrations of these drugs in the aqueous phase, quoted for these *in vitro* experiments, may bear rather little relationship to the actual effective (and time-variable) concentrations of these drugs within the tissue and available to inhibit the autolytic enzyme, presumed to be within the chondrocytes.

Drug action on chondromucoprotease

The chondromucoprotease described here was difficult to characterize apart from its sensitivity to antimalarials. We could not show its identity with any known

extracellular proteases or cathepsins; but our failure to do so, might merely reflect the fact that potential protease inhibitors used in this study and commonly employed to diagnose sulphydryl-enzymes or serine-enzymes, penetrated the cartilage tissue too slowly or in insufficient quantity to block the chondromucoprotease. Our lack of success to date in obtaining reasonably stable and consistently active cell-free preparations of the enzyme also hindered its characterization. All the evidence currently available suggests that it is not identical with trypsin or chymotrypsin in substrate specificity or drug sensitivity. In its acid pH optimum and certain other properties, this bovine chondromucoprotease certainly resembles other recently described autolytic enzymes present in various cartilaginous tissues,[22] human articular cartilage,[23] rabbit ears[24] and embryonic chick limb cartilage.[25] However the bovine enzyme differs from the three latter cartilage-degrading enzymes in being almost insensitive to cyanide, 6-aminohexoic acid and vitamin A (see Refs. 23–26 respectively).

The question then arises, do these antimalarials inhibit this chondromucoprotease by stabilizing lysosomal particles in cartilage-assuming that this is indeed a lysosomal enzyme? Lysosomal enzymes commonly exhibit their greatest activity at pH 5 and may be activated or released by vitamin A, streptolysin[27] etc. Chloroquine stabilizes rabbit and rat liver lysosomes preventing the release of catheptic/hydrolytic enzymes after injury etc.[27, 28] However, it is reported[28] that chloroquine does not protect rat liver lysosomes from disruption at pH 5.

If this drug can so stabilize an intracellular membrane, might it not also stabilize other membranes (not necessarily part of the lysosome wall) which normally prevent the chondromucoprotease from coming into contact with its substrate? This substrate is probably extracellular material.

We could obtain no good evidence that the chondromucoprotease was a lysosomal enzyme. Its activity was not significantly changed by pretreating cartilage slices with reagents or conditions known to either disrupt lysosomes (e.g. vitamin A at various pH's, freezing and thawing, neutral detergents) or to stabilize lysosomes (e.g. hydrocortisone). The meagre cell-free enzyme activity which could be extracted from cartilage homogenates was certainly chloroquine-sensitive but since this represented only a small fraction of the original autolytic activity, we cannot infer that *all* the chloroquine-sensitive chondromucoprotease activity of intact cartilage is therefore extra-lysosomal.

It was surprising to find that cartilage could metabolize chloroquine and hydroxychloroquine to a limited extent. The same metabolites of these drugs have been found in spleen, eyes and other extrahepatic tissues.[29] The first-formed metabolites of these drugs (i.e. secondary and primary amines) were approximately equipotent with the drugs being metabolized in their ability to inhibit both the chondromucoprotease and the collagenases.

Drug action on other enzymes

This fact that these antimalarial drugs inhibited both a bacterial collagenase (at pH 7) and a skin collagenase (at pH 5) may be rather less significant than their potential drug action on the chondromucoprotease. This drug action was on the collagenase enzymes, since gelatine degradation by these collagenases was drug-sensitive but gelatine degradation by papain and trypsin was not [the parallel findings that cartilage or chondromucoprotein degradation by papain (or trypsin) and hyaluronidase was

also drug insensitive would suggest that the antimalarials were not just "blanketing" the endogenous chondromucoprotein substrate to protect it from all exogenous enzymes, but selectively acting on the chondromucoprotease].

The bacterial collagenase, clostridiopeptidase A, was sensitive to many different organic bases, particularly to certain aminoacridines and quaternary detergents (cetylamines). The latter compounds are well-known bacteriostats but they would also seem to be particularly valuable for protecting connective tissues from the consequences of a clostridial infection (e.g. "gas gangrene") if applied topically. They are certainly more potent than most of the compounds known to inhibit the bacterial collagenases.[30]

Chloroquine binds to sulphydryl groups on proteins at pH's above 7 and may suppress chemical reaction involving the thiol group of cysteine at pH 4·5.[31] It is notable that chloroquine failed to inhibit papain action when this sulphydryl enzyme[32] was activated by cyanide. High levels of chloroquine did however inhibit a cartilage esterase, which was apparently a sulphydryl enzyme. Thus it is apparent that chloroquine might inhibit several hydrolytic enzyme activities in cartilage, some of which are sulphydryl-dependent (e.g. this esterase) while others are insensitive to the standard sulphydryl reagents but nevertheless chloroquine-sensitive (e.g. chondromucoprotease).

Chloroquine as an anti-arthritic drug

Intra-articular administration of chloroquine is reported to be of value in the treatment of polyarthritis and degenerative arthritis.[33] In osteoarthritis, acidic mucopolysaccharides are lost from the articular cartilage (chondromalacia).[34] This might arise through (a) activation of the endogenous chondromucoprotease through reduction of the pH in a joint, (b) action of exogenous proteases such as plasmin or leukocyte cathepsins or (c) mucopolysaccharase action (e.g. hyaluronidase).[35] These experiments with bovine non-articular cartilage indicate that chloroquine might be expected to inhibit only the chondromucoprotease, assuming that human articular cartilage is not too different from bovine nasal and tracheal cartilage (or rabbit ear cartilage) in its content of autolytic enzymes and drug- and enzyme-susceptibility. Weissman's finding[27] that chloroquine may stabilize lysosomes suggests that this drug might also prevent the release of proteolytic (and chondrolytic) enzymes from leukocytes or synovial cells in an inflamed joint.

A form of experimental arthritis with cartilage dystrophy is produced when papain is injected into knee joints[36] or administered intravenously[37] in rabbits and monkeys. Since papain releases chondromucopeptides from many different types of cartilage, it is highly probable that the autolytic chondromucoprotease (if activated) which also releases chondromucopeptides, would cause similar dystrophy (arthritis) to that induced experimentally with papain.

Simultaneously with the loss of polysaccharides from the matrix in cartilage erosion and osteoarthritis, there is increased biosynthesis of sulphated polysaccharides by the chondrocytes[38] in response to the dissolution of the matrix in their vicinity. Together, these opposed processes of matrix lysis and regeneration may lead to an uneven joint surface as is often found in chronic joint inflammation. Chloroquine also inhibits this regenerative process at reasonably low levels (1 mM.)[1] Therefore it does seem to be a rather suitable drug, on theoretical grounds, for minimizing both cartilage erosion (including collagenolysis) and also the consequent repair processes which,

if uncontrolled, could cause further joint malformation. (Mepacrine would seem even more suitable, being more potent than chloroquine in both respects).

Like other anti-inflammatory/antirheumatic drugs,[39] chloroquine may then owe its therapeutic value to no single biochemical property but act as a polyvalent drug inhibiting several different enzyme systems—including some which cause tissue destruction (as demonstrated here) and others underlying unwonted (and unwanted?) tissue proliferation. Chloroquine is known to inhibit nucleic acid biosynthesis[40] and might therefore be expected to limit tissue proliferation or regeneration to a certain degree.

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