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THE EFFECT OF ANTI-INFLAMMATORY DRUGS ON THE ENZYMIC ACTIVITY OF A RAT LIVER GRANULAR FRACTION WHICH INCREASES VASCULAR PERMEABILITY

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Abstract—The inhibition by anti-inflammatory drugs of testicular hyaluronidase, and of lysosomal acidic hydrolases present in a rat liver granular fraction, was investigated *in vitro*. At concentrations needed in plasma for anti-inflammatory effects in man, the clinically useful nonsteroidal anti-inflammatory drugs phenylbutazone, flufenamic and mefenamic acids, indomethacin and ibufenac inhibited hyaluronidase and β-glucuronidase, while aspirin inhibited acid phosphatase and cathepsins. It is suggested these drugs conserve the integrity of cartilage and other connective tissue chondromucoprotein *in vivo* by inhibiting lysosomal enzymic attack on either the mucopolysaccharide or protein moiety.

Permeability factors detected in the granular fraction indicate that, like lysosomal enzymes, they exist latent *in vivo* within intact sub-cellular particles, and are liberated in an active form only after disruption of the particle membrane. The implications of these factors in inflammation, and their possible relationship to lysosomal enzyme activity and drug action, are discussed.

MANY anti-inflammatory drugs activate ATP breakdown and inhibit oxidation and phosphorylation reactions, thereby affecting ATP-dependent enzymic reactions carried out at subcellular levels.[1]

Little is known, however, of the effect of these drugs on enzymes which depolymerise connective tissue components, and which appear active in inflammatory diseases such as rheumatoid arthritis in which chondrolysis often occurs. The effect of some analgesic, anti-pyretic and anti-inflammatory drugs on the activities of some degradative acid hydrolases has therefore been investigated. There is increasing evidence that these enzymes, normally latent within sub-cellular lysosomes,[2] are released in an active form during certain experimental tissue injuries[3] and human diseases,[4–6] thereby contributing to the inflammatory responser

A rat liver granular fraction, used in this study as a soures of lysosomal enzymes, was found to increase vascular permeability when injected intradermally into rats. Some properties of these permeability factors and their possible relationship to lysosomal enzymatic activity, inflammation and drug action, are discussed.

MATERIALS AND METHODS

Chemicals and drugs

Chondroitin sulphate and hyaluronidase (ex bovine testes) were bought from Koch-Light Laboratories Ltd. Trypsin ('Tryptar') was bought from Armour Pharmaceutical Company Ltd. Triton X-100 was a gift from Lonnig Chemicals Ltd.

Pontamine Sky Blue (16 BX) was bought from Edward Gurr Ltd. Chondromucoprotein was fraction A isolated from human cartilage.[7] Methisergide bimaleate and mepyramine maleate ('Anthisan') were bought from Sandoz Products Ltd. and May & Baker Ltd., respectively.

The following drugs were investigated: aspirin (Monsanto Chemicals Ltd.), phenylbutazone (Geigy Pharmaceutical Industries), flufenamic acid (Parke, Davis & Co.), ibufenac (Research Department, Boots Ltd.), chloroquine (Sigma Chemical Company), 4-aminophenazone (Hopkin & Williams Ltd.), D-penicillamine (Lilly Research Laboratories Ltd.), mefenamic acid (Parke, Davis & Co.), adrenaline (British Drug Houses Ltd.) and indomethacin (Merck Sharp & Dohme Ltd.).

The drugs were dissolved or suspended in their appropriate solvent within 1 hr before they were tested. Aspirin, phenylbutazone, flufenamic acid and ibufenac were dissolved in absolute ethyl alcohol. Chloroquine, 4-aminophenazone and D-penicillamine were dissolved in water. Mefenamic acid and adrenaline were suspended in 0·05 N-NaOH and 0·03 N-HCl, respectively, while indomethacin was suspended in absolute ethyl alcohol. Stated concentrations of the drugs refer to their final concentrations in the enzyme mixtures. All the drugs were soluble at concentrations of 2 mM, except phenylbutazone and flufenamic acid, which were soluble at 0·05 mM, and mefenamic acid and indomethacin, which were soluble at 1 mM.

Solutions

Saline. This was 0·9% (w/v) sodium chloride in water.

Pontamine Sky Blue solution. This was a solution (5%, w/v) in saline.

Sucrose solution. Sucrose was dissolved in 0·05 M-Tris buffer, pH 7·4, to a concentration of 0·25 M.

Phenolphthalein-β-glucuronide substrate. The sodium salt (Koch-Light Laboratories Ltd.) was used at a concentration of 0·1% (w/v) in water.

Glycine solution. A solution of 7·5 g of glycine in 100 ml of water was adjusted to pH 10 with 1 N-NaOH. This solution was made up to 250 ml with water.

β-Glycerophosphate substrate. This was prepared by dissolving 3·15 g of the sodium salt (Koch-Light Laboratories Ltd.) and 8·5 g of sucrose in 50 ml of 0·2 M-acetate buffer, pH 5. Glacial acetic acid was added to pH 5, and the volume made up to 100 ml with water.

Molybdate solution. This was a mixture of 250 ml of 2·5% (w/v) ammonium molybdate and 125 ml of 60% (v/v) perchloric acid.

Ascorbic acid solution. A solution of ascorbic acid (0·1%, w/v) was prepared immediately before use.

Casein solution. A solution of light white soluble casein (British Drug Houses Ltd.) was used, at a concentration of 4% (w/v) in 6 M-urea.

Albumin solution. This was 1% (w/v) bovine serum albumin (Koch-Light Laboratories Ltd.) in 0·05 M-acetate buffer, pH 4.

Preparation of rat liver granular fraction

Livers, removed from rats killed with nitrous oxide, were cut into small pieces with scissors and washed several times with chilled sucrose solution. Portions (2 g) were homogenized at 4° in a Potter-type homogenizer with 8 ml of sucrose solution (5 up-and-down strokes). The homogenates obtained from 10 g of liver were

pooled, and after the addition of sucrose solution to make a 10% (w/v) suspension, the total homogenate was subjected to differential centrifugation in a Mistral M.S.E. centrifuge at 4° in an angle rotor (no. 69404, max. radius 3·5 in.). Nuclei and debris were centrifuged down at 600 *g* for 5 min and rejected. The resulting supernatant fluid was centrifuged at 15,000 *g* for 20 min, and the sedimentated granular fraction, containing mitochondria and lysosomes, was carefully washed twice with sucrose solution. The sub-cellular particles were then disrupted by suspending them in 20 ml of 0·1% (v/v) Triton X-100 for 30 min at 4°. The suspension was then centrifuged at 15,000 *g* for 20 min to remove debris, the resulting supernatant fluid retained, and the pellet washed twice with 20 ml of Triton solution. To the combined supernatant fluid and washings was added solid NaCl to 0·9% (w/v), and the solution adjusted to pH 4·6 with 0·1 N-HCl. After removal of insoluble material by centrifugation, the resulting supernatant fluid was dialysed exhaustively against distilled water at 4° and the non-diffusible material freeze-dried. Yield: 6 mg of granular fraction/g liver.

Enzyme assays

Extinction values were measured in cells of 1 cm lightpath in a Unicam (SP 500) spectrophotometer.

β-glucuronidase. This was estimated at pH 5, using phenolphthalein-β-glucuronide as substrate. Additions were made in the following order: 0·05 ml of drug solution; 0·7 ml of 0·1 M-acetate buffer, pH 5; 0·3 ml of granular fraction (0·1 mg) in saline; 0·1 ml of substrate. A control contained the same ingredients except that 0·05 ml of solvent was added in place of the drug. In the absence of granular fraction, the substrate and drug alone did not affect the assay. The tubes were incubated at 37° for 1 hr and the liberated phenolphthalein measured by the method of Gianetto and de Duve.[8] Results were expressed as percentage reduction of extinction values in the presence of the drug, compared with the control.

Acid phosphatase. This was estimated at pH 5, using β-glycerophosphate as substrate. Additions were made in the following order: 0·05 ml of drug solution; 0·2 ml of granular fraction (0·1 mg) in saline; 0·3 ml of substrate. A control contained the same ingredients, except that 0·05 ml of solvent was added in place of the drug. A reagent blank contained the same ingredients as the control, except that 0·2 ml of saline was included in place of the granular fraction, to allow for colour contribution by the substrate. The tubes were incubated at 37° for 1 hr and the liberated phosphate was measured by the method of Gianetto and de Duve.[8] After the appropriate corrections, results were expressed as percentage reduction of extinction values in the presence of the drug, compared with the controls.

Cathepsins. These were estimated at pH 5, using casein as substrate. Additions were made in the following order (series T): 0·1 ml of drug solution; 1·7 ml of 0·2 M-acetate buffer, pH 5: 0·5 ml of granular fraction (0·5 mg) in saline; 1 ml of casein solution. A control was included (series B), which contained the same ingredients as series T, except that 0·1 ml of solvent was added in place of the drug. A reagent blank contained the same ingredients as the control (series B), except that 0·5 ml of saline was included in place of the granular fraction. After incubation at 37° for 1 hr the acid-soluble peptides released were estimated by the method of Ali and Evans.[9] To compensate for absorption at 280 mμ by some drugs (aspirin, 4-aminophenazone, chloroquine and adrenaline) series X was included which was identical with series T, except that

trichloracetic acid was added immediately after the addition of the casein solution. After the appropriate corrections, results were expressed as percentage reduction of extinction values in the presence of the drug, compared with the controls.

Hyaluronidase. This was estimated at pH 4, using chondroitin sulphate as substrate. A method was developed which relied on the fact that, at low ionic strength, mucopolysaccharides form a stable colloidal suspension with albumin at pH 4, due to formation of insoluble mucopolysaccharide-albumin complexes. These can be measured turbidimetrically.[10] Mucopolysaccharides depolymerized by hyaluronidase progressively lose this ability, enzyme activity thus being inversely proportional to the extent of complex formation of the mucopolysaccharide with albumin. Additions were made in the following order, using in each case 0·1 M-acetate buffer, pH 4, containing 0·15 M-NaCl: 0·05 ml of drug solution; 1 ml of testicular hyaluronidase (0·05 mg) in buffer; 1 ml of chondroitin sulphate (0·5 mg) in buffer. A reagent blank was included which contained the same ingredients, except that 0·05 ml of solvent was added in place of the drug. To determine the turbidity formed by undepolymerized chondroitin sulphate, a control (A) was included which contained 0·05 ml of solvent, 1 ml of buffer and 1 ml of chondroitin sulphate (0·5 mg) in buffer. After incubation at 37° for 1 hr the mixtures were centrifuged, 0·4 ml of each supernatant fluid added to 2 ml of albumin solution and turbidities due to precipitated mucopolysaccharide-albumin complexes measured at 400 mμ. Results were expressed as percentage reduction in turbidities, compared with control A. The same method was used to estimate hyaluronidase in the granular fraction, except that 1 ml of granular fraction (1·5 mg) was used in place of testicular hyaluronidase (0·05 mg).

Depolymerization by testicular hyaluronidase of chondroitin sulphate and chondromucoprotein

The following additions were made to a series of tubes, using 0·1 M-acetate buffer, pH 4, containing 0·15 M-NaCl: 1 ml of chondroitin sulphate (0·5 mg) in buffer; 1 ml of testicular hyaluronidase (containing 0–0·2 mg) in buffer. A control tube contained 1 ml of chondroitin sulphate (0·5 mg) in buffer and 1 ml of buffer. A second series of tubes contained 1 ml of chondromucoprotein (0·5 mg) in buffer and 1 ml of testicular hyaluronidase (0–0·2 mg) in buffer. A control tube contained 1 ml of chondromucoprotin (0·5 mg) in buffer and 1 ml of buffer. After incubation at 37° for 1 hr, the contents of each tube were centrifuged and 0·4 ml of each supernatant fluid added to 2 ml of albumin solution. Turbidities were measured at 400 mμ and the results expressed as percentage reductions in turbidities compared with the turbidity of the appropriate control.

Depolymerization by testicular hyaluronidase of chondromucoprotein, following the proteolytic removal of the protein moiety

Trypsin digestion was carried out in 0·05 M-phosphate buffer, pH 8. Into a tube was placed 0·8 ml of chondromucoprotein (10 mg) in buffer, followed by 0·2 ml of trypsin (1·0 mg) in buffer. A control tube contained 10 mg of chondromucoprotein in 1 ml of buffer. Both tubes were incubated at 37°. Portions (0·1 ml) were removed at 0, 1, 4 and 24 hr and added to 0·9 ml of 0·1 M-acetate buffer, pH 4. After adding 1 ml of testicular hyaluronidase (0·5 mg) in acetate buffer, the tubes were incubated at 37° for 1 hr. Portions (0·3 ml) were then added to 2 ml of albumin solution and the

turbidities which formed were measured at 400 mμ. Results were expressed as percentage reduction in turbidities, compared with the control.

Vascular permeability experiments

Wistar rats (100–130 g) were anaesthetized with ether and injected i.v. into the tail with 0·2 ml of Pontamine Sky Blue solution, followed immediately by the intradermal injection into the shaved abdomen of 0·1 ml of the test solution. Injection of 0·1 ml of saline into the rat served as the control. Some rats were killed after 45 min, their skins stripped off and the degree of blueing on the inner surface assessed by visual inspection. Some rats were killed after 24 hr. Skin sections were prepared and stained with haematoxylin and eosin.

The histamine antagonist, mepyramine maleate, and the 5-HT antagonist, methisergide bimaleate, were injected i.v. (2 mg/kg, in saline) immediately after the injection of Pontamine Sky Blue.

RESULTS

The inhibition of β-glucuronidase, testicular hyaluronidase, acid phosphatase and cathepsins by the drugs investigated, at final concentrations of 2 mM, is shown in Table 1, the inhibition of these enzymes by the more potent of these drugs being

TABLE 1. THE *IN VITRO* INHIBITION BY DRUGS AT 2mM OF TESTICULAR HYALURONIDASE AND LYSOSOMAL ENZYMES DERIVED FROM A RAT LIVER GRANULAR FRACTION

Drug	Inhibition (%)				
	β-glucuronidase	hyaluronidase		acid phosphatase	cathepsins
		testicular	granular fraction		
Aspirin	2	0	1	67	29
phenylbutazone	75	47	8	14	0
4-aminophenazone	4	0	0	10	0
chloroquine	19	0	0	—	2
flufenamic acid	15	70	8	30	7
mefenamic acid	10	74	4	—	6
indomethacin	51	29	9	33	0
ibufenac	9	48	24	7	17
adrenaline	9	0	0	—	0
D-penicillamine	9	0	1	—	2

detailed in Figs. 1 to 4, respectively. The inhibition of granular fraction hyaluronidase by ibufenac, using chondroitin sulphate as substrate, is shown in Fig. 2.

The depolymerization by testicular hyaluronidase of chondroitin sulphate and chondromucoprotein is shown in Fig. 5. The depolymerization by testicular hyaluronidase of chondromucoprotein, after the progressive removal of the protein moiety with trypsin, is shown in Fig. 6.

Vascular permeability studies

On heating a saline solution of the granular fraction at 100° for 10 min the activities of β-glucuronidase, hyaluronidase, acid phosphatase and cathepsins were decreased

by 99, 57, 90 and 90 per cent, respectively. After heating at 100° for 30 min the activity of hyaluronidase was decreased by 64 per cent. Suspensions of 1, 0·5, 0·25 and 0·125 mg of granular fraction in 0·1 ml of saline produced graded blueing when injected intradermally into rats, the response with 0·5 mg corresponding to 1 μg of histamine. Insoluble material obtained after centrifuging these suspensions (which

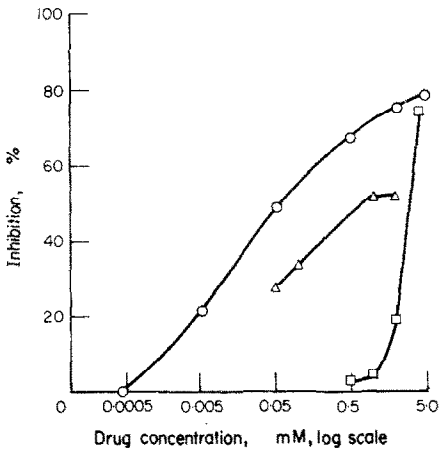

FIG. 1. The inhibition of rat liver granular fraction β-glucuronidase by: ○, phenylbutazone; △, indomethacin; □, chloroquine. Experimental details are given in the text.

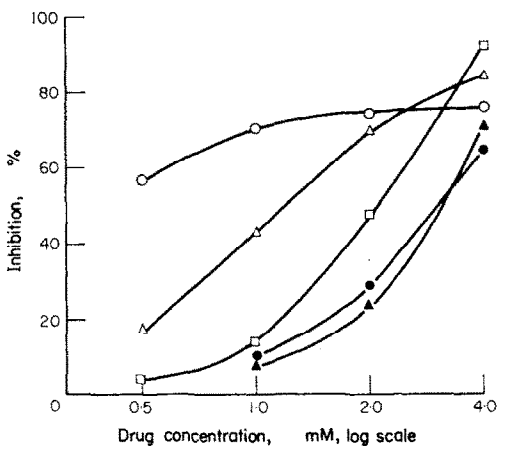

FIG. 2. The inhibition of testicular hyaluronidase by: ○, mefenamic acid; △, flufenamic acid; □, phenylbutazone; ●, indomethacin. The inhibition of rat liver granular fraction hyaluronidase by ibufenac is shown by '▲'. Experimental details are given in the text.

amounted to 26 per cent by weight) was inactive. Heating at 100° decreased the activity slightly after 10 min and appreciably after 30 min, precipitated material being inactive. Activity was unaffected by adjusting granular fraction solutions to pH 4 and pH 8, followed before injection by re-adjustment to pH 7. These treatments did not form dialysable material which could increase vascular permeability.

When the granular fraction was injected with sodium salicylate at 160 mM, vascular permeability was completely suppressed. At 20 mM partial suppression occurred, slight suppression being detected at 2 mM. The increase in vascular permeability following the intradermal injection of the granular fraction was partially suppressed by the prior i.v. injection of the histamine antagonist and, to a greater

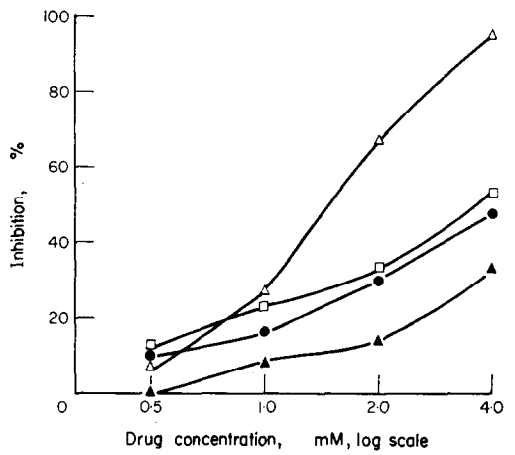

FIG. 3. The inhibition of rat liver granular fraction acid phosphatase by: △, aspirin; □, indomethacin; ●, flufenamic acid; ▲, phenylbutazone. Experimental details are given in the text.

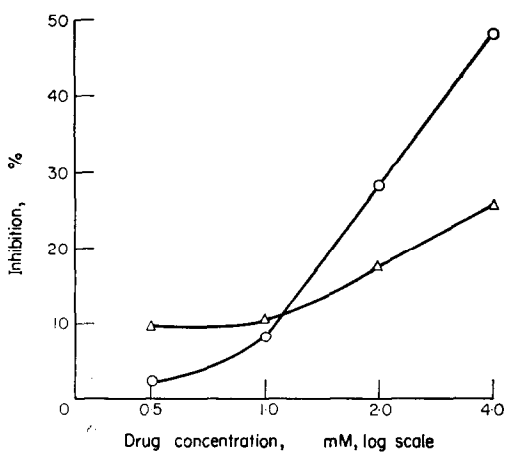

FIG. 4. The inhibition of rat liver granular fraction cathepsins by: ○, aspirin; △, ibufenac. Experimental details are given in the text.

extent, by the 5-HT antagonist. In a subsidiary experiment, the intravenous injection of rats with these antagonists completely suppressed the response following the intradermal injection of 1 μg of histamine and 2·5 μg of 5-HT, respectively.

The absorption spectrum of a solution of the granular fraction in saline (0·67%, w/v) contained a broad peak with a maximum at 274 mμ, which decreased to 271 mμ after heating at 100° for 10 min.

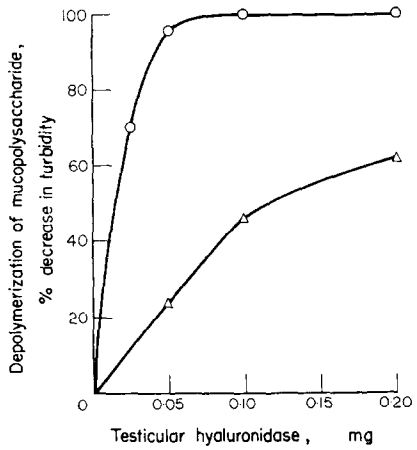

FIG. 5. Depolymerization of chondroitin sulphate '○' and chondromucoprotein '△' by testicular hyaluronidase. The mucopolysaccharide was incubated at pH 4 with increasing amounts of testicular hyaluronidase for 1 hr at 37°. Mucopolysaccharide, in absence of enzyme, served as the control. The ability of the mucopolysaccharide to form a turbidity with albumin at pH 4 was measured. This was inversely proportional to the extent of depolymerization, and therefore of hyaluronidase activity. Results were expressed as percentage decrease in turbidity compared to the control.

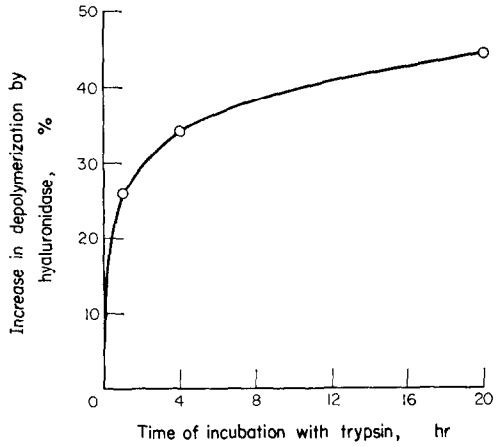

FIG. 6. Depolymerization by testicular hyaluronidase of chondromucoprotein, following proteolytic removal of the protein moiety. Chondromucoprotein was incubated with trypsin at pH 8. At various time intervals aliquots were removed, and the ability of testicular hyaluronidase to deploymerize the resulting chondromucopeptides determined.

Histologically, little change could be detected at the injection site after 45 min. After 24 hr, however, mononuclear cells had infiltrated, and considerable amounts of amorphous eosinophilic 'fibrinoid' material had deposited.

DISCUSSION

Due to their high molecular weight, linearity and anionic character, the acidic mucopolysaccharides maintain the integrity of many biological fluids and connective tissues, promoting high viscosity and lubrication properties, ion balance and hydration.

Chondromucoprotein, which is the naturally occurring form of chondroitin sulphates A and C in cartilage,[11] in which the mucopolysaccharides are linked covalently to non-collagenous material which is probably glycoprotein,[7, 12] is involved in the formation of collagen and probably in the maintenance of its ordered structure *in vivo*.[13] Depolymerization of these substances *in vivo* results in the loss of these properties, the formation of low molecular weight diffusible residues and tissue disorganization.

Enzymes which degrade mucopolysaccharides include hyaluronidase, which depolymerizes hyaluronic acid and chondroitin sulphates A and C to oligosaccharides,[14] and β-glucuronidase which causes further degradation to sulphated trisaccharides and free glucuronic acid.[15] Chondromucoprotein is depolymerized *in vitro* and *in vivo* either by proteolysis of the protein moiety by trypsin, papain or plasmin to form chondromucopeptides, [7, 16–19] or by degradation of the chondroitin sulphate moiety by hyaluronidase and β-glucuronidase to form protein-oligosaccharide units.[20]

Attention has centered recently on hydrolytic enzymes normally latent within lysosomes, but capable when released of degrading protein, nucleic acids and mucopolysaccharides at pH values below 5.[2, 21, 22] These enzymes also occur in macrophages,[23] polymorphonuclear leucocyte granules[24] and other circulating cells which migrate to inflammatory sites following increased vascular permeability. Their release following cell degeneration has been implicated in the connective tissue changes following exposure of skin to u.v. light, injection of streptolysin S into joints, hypervitaminosis A, endotoxin shock, tissue necrosis, connective tissue diseases and autoimmune phenomena.[3–6, 25] Recent work suggests that the physiological pH at the surface of connective tissue cells and macromolecules *in vivo*, particularly at anoxic inflammatory sites, is approximately 5 rather than the classical 7, and therefore sufficiently low for significant lysosomal enzymic activity.[26–29]

Rheumatoid synovial fluid, which has a low viscosity due to a decreased hyaluronic acid content,[30] also contains acid phosphatase, β-glucuronidase and other lysosomal enzymes[31, 32] which probably originate from infiltrated degenerating leucocytes[33] and damaged synovial tissue.[34] Rheumatoid synovial tissue itself contains a higher level of these enzymes than normal synovia.[35] They are also present in lesions taken from normal rabbits injected intradermally with heat-killed bacteria, although not from leucopenic rabbits.[36] Also, cortisone and other anti-rheumatic steroids stabilize lysosomal membranes *in vitro*,[37] while vitamin A, which is chondrolytic in rabbits, disrupts membranes in organ culture.[38]

Results of the present study strengthen the relevence of lysosomes to the problem of inflammation. Phenylbutazone, flufenamic and mefenamic acids, indomethacin and ibufenac inhibited hyaluronidase and β-glucuronidase at concentrations approximating to plasma levels needed for anti-inflammatory activity in man.[39] These acidic lipophilic drugs are the most useful non-steroidal anti-rheumatic agents,[40–46] and may act *in vivo* by limiting depolymerization of the chondroitin sulphate moiety of cartilage chondromucoprotein and synovial fluid hyaluronic acid.

With the exception of ibufenac, these drugs were less efficient inhibitors of granular fraction hyaluronidase than of testicular hyaluronidase, despite the similarity of these two enzymes.[47, 48] However, only 0·01 mg of lysosomal hyaluronidase is present in 1 g of rat liver,[47] and in this study 1·5 mg of granular fraction (obtained from 0·25 g of liver) was needed in the hyaluronidase assay compared with 0·05 mg of testicular

hyaluronidase (see 'Materials & Methods' section). Further studies on the inhibition of granular fraction hyaluronidase by drugs would require a fraction with a higher specific activity.

Hyaluronidase depolymerized free chondroitin sulphate more readily than chondromucoprotein (Fig. 5). This was probably due to steric hindrance by the protein moiety since its removal by trypsin yielded chondromucopeptides more susceptible to hyaluronidase action (Fig. 6). Proteolytic digestion of cartilage chondromucoprotein *in vivo* by lysosomal cathepsins may therefore be a contributory factor for attack on the chondroitin sulphate moiety by hyaluronidase and β-glucuronidase.

At concentrations needed in plasma for an anti-inflammatory effect in man[49], aspirin was the most potent inhibitor of lysosomal cathepsins, and would therefore help to maintain *in vivo* the integrity of chondromucoprotein by inhibiting breakdown of the protein moiety. The clinical effectiveness of aspirin in combination with an inhibitor of the mucopolysaccharidases discussed above could theoretically be due to prevention of attack on cartilage chondromucoprotein by both lysosomal cathepsins and mucopolysaccharidases.

Although the role of acid phosphatase in inflammation is unknown, it is present in raised concentration in rheumatoid synovial fluid[50] and inhibited by aspirin.

Adrenaline, D-penicillamine and 4-aminophenazone had no effect on the enzymes investigated. The effectiveness of D-penicillamine in rheumatoid arthritis is uncertain, although improvement has been claimed.[51, 52] Chloroquine, which relieves rheumatoid symptoms after prolonged administration,[53] inhibited β-glucuronidase only at concentrations of 4 mM and above.

It is unlikely that the permeability factors described in this study are peculiar to rat liver granular fraction, since similar factors are present in rat lymph node extracts, subcellular particles from other rat tissues, leucocytes and macrophages.[54–56] The fact, however, that these factors were only released, as were the lysosomal enzymes, in an active form when sub-cellular particles in the granular fraction were disrupted with Triton (see 'Materials & Methods' section), suggest they contribute to increased vascular permeability at inflammatory sites where cell infiltration, followed by degranulation, has occurred.

With the exception of hyaluronidase, lysosomal enzymes in the heat-treated granular fraction were inactive and therefore did not contribute to the observed increase in permeability. However, increased vascular permeability caused by heat-treated granular fraction would result in local extra-vascular accumulation of dye, followed by extensive diffusion around the injection site due to increased tissue permeability arising from depolymerization of ground substance by hyaluronidase. Active lysosomal enzymes in the untreated granular fraction may also contribute to this initial response. Salicylates, which inhibit hyaluronidase at 250 mM *in vitro*[57] and *in vivo*,[58] and also lysosomal cathepsins *in vitro* at 4 mM (Fig. 4), completely suppressed the blueing reaction at 160 mM. At a later stage in the permeability response, it is suggested that degranulation of infiltrated cells would augment the inflammatory response due to release of additional permeability factors and lysosomal enzymes, while mast cells, perhaps degranulated by a cell-rupturing agent present in leucocyte lysosomes,[59] would liberate the permeability factors histamine and 5-HT. In the present study it was evident that these permeability factors were liberated *in vivo* since injection of rats with pharmacological antagonists reduced the blueing reaction.

'Fibrinoid' material seen twenty four hours after the injection of granular fraction may consist in part, as suggested elsewhere,[60] of insoluble complexes formed by electrostatic interaction between anionic ground substance components, which would include nucleic acids and mucopolysaccharides, and extra-vascular plasma proteins existing as cations at the inflammatory site where the pH is below their isoelectric points.

It is possible that the acidic non-steroidal anti-inflammatory drugs at present in use function, in the sequence of events postulated above, solely as lysosomal enzyme inhibitors. The possibilities that these drugs may stabilize lysosomal membranes and thus prevent the release of enzymes, as does chloroquine and certain steroids, or may even enter lysosomes and inhibit the enzymes *in situ*, must be considered.

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