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STUDIES ON THE RELEASE OF PROTEOLYTIC ENZYMES DURING SYNOVIUM-INDUCED CARTILAGE BREAKDOWN *IN VITRO* AND THE ACTIONS OF ANTI-INFLAMMATORY DRUGS

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Abstract—Pig articular cartilage, overlaid with a minced preparation of synovium from the same joint, underwent extensive matrix degradation during a two-week culture period. This degradation was associated with *de novo* synthesis by the synovium of specific neutral proteoglycan- and collagen-degrading enzymes. Both enzymes exhibited neutral pH optima, and were inhibited by serum and the metal ion chelators EGTA and 1,10-phenanthroline. The neutral proteoglycanase cleaved the core protein of isolated proteoglycan. The effects of some anti-inflammatory drugs on synovial enzyme production and cartilage metabolism were investigated. The steroids, dexamethasone and prednisolone, inhibited production of both enzymes whereas the non-steroidal anti-inflammatory drugs (NSAID's), flurbiprofen and indomethacin, slightly increased medium enzyme levels. Flurbiprofen and indomethacin had no effect on the extent of synovium-mediated cartilage degradation as assessed histologically. Inhibition by the steroids of synovial collagenase production correlated with inhibition of cartilage collagen breakdown, whereas inhibition of synovial proteoglycanase production did not prevent extensive proteoglycan breakdown. Experiments using radiotracer techniques indicated that dexamethasone, whilst partially inhibiting synovium-mediated proteoglycan degradation, severely inhibited cartilage proteoglycan synthesis thus resulting in net proteoglycan loss.

The chronic inflammatory lesion of rheumatoid arthritis is characterised by synovial lining cell hyperplasia and an accumulation within the joint of large numbers of inflammatory cells, particularly lymphocytes and macrophages.

It is widely believed that the destruction of joint connective tissues, particularly cartilage, in rheumatoid arthritis is mediated, at least in part, by degradative enzymes secreted by cells of the inflammatory synovial tissue (for review see Krane [1]).

Using an organ culture system consisting of slices of living articular cartilage, obtained from the metacarpophalangeal joints of young pigs, overlaid with a minced preparation of synovial tissue from the same joint, Fell and Jubb [2] demonstrated that the cartilage became depleted of both proteoglycan and collagen during a 14-day culture period. From investigations using living and dead cartilage cultured in contact with or removed from either living or dead synovium, they suggested that cartilage matrix breakdown was mediated by two separate mechanisms, i.e. direct attack by presumably synovial enzymes or by release of synovial catabolic factors which stimulated living chondrocytes to degrade their own matrix. A factor, termed catabolin, has recently been identified in culture media of porcine synovium and partially characterised [3, 4].

Using the pig-cartilage/synovium co-culture system of Fell and Jubb [2], with slight modifications, we have demonstrated that the synovial tissue synthesises and secretes specific proteoglycan- and collagen-degrading enzymes. Partial characterisation of both these enzymes is described. In addition we have examined the effects of some steroidal and non-steroidal anti-inflammatory drugs on synovial enzyme production and cartilage metabolism. The effects of these drugs *in vitro* are discussed in relation to their modes of action *in vivo*.

MATERIALS AND METHODS

Cartilage and synovium cultures. The preparation and culture of pig articular cartilage and synovium was essentially as described by Fell and Jubb [2] with slight modifications. Articular cartilage and synovium were obtained from the metacarpophalangeal joints of young pigs. Cartilage slices (5 mm in length and 1 mm thick) were placed, articular surface uppermost, onto Millipore filters (13 mm diameter; pore size 0.2 μm) supported on stainless steel mesh grids in small plastic petri dishes (30 mm diameter, Sterilin). Preliminary experiments had indicated that slices of these dimensions had similar DNA content. Synovial tissue from a number of joints was pooled, minced finely with scissors, and 0.1 ml aliquots were dispensed over half the length of the cartilage explants. Each petri dish contained 1.5 ml Dulbecco's modified Eagles medium, DMEM (Flow laboratories), containing ascorbic acid, 50 μg/ml, glutamine 580 μg/ml, benzylpenicillin 500 μg/ml and streptomycin sulphate 500 μg/ml. Medium was supplemented with heat-inactivated, acid-denatured rabbit serum to a final concentration of 5% v/v. Rabbit serum, obtained from animals kept in-house, was sterilised by pressure filtration through 0.22 μm millipore membranes, heat-inactivated at 56° for 30 min to destroy complement, and acid-denatured as des-

cribed previously [5], to destroy serum proteinase inhibitors [6]. Culture dishes were incubated in Fildes–Macintosh jars at 37° in a 5% CO_2/95% air atmosphere. Media were harvested and replenished at 2–3 day intervals for periods up to 14 days. Harvested media were stored at −20° prior to analysis. All procedures involving tissue preparation and harvesting of media were carried out under sterile conditions in laminar flow cabinets at room temperature. In stated experiments, drugs were dissolved in dimethyl sulphoxide (DMSO) 10^{-1} M and further diluted with DMEM/5% rabbit serum to required concentrations. Drugs were included throughout the culture period. Control cultures contained the appropriate concentrations of DMSO which has been found to have no significant effect on cartilage breakdown.

Histology. Tissues for histological examination were fixed in formal saline prior to sectioning. For visualisation of proteoglycan and collagen content sections were stained with *o*-toluidine blue and Van Gieson's stain/Carazzi's haematoxylin/celestine blue respectively.

Chondroitin sulphate assay. Chondroitin sulphate levels of cartilage digests were assayed by the method of Whiteman [7] except that the alcian blue/proteoglycan complex was solubilised in 0.05 M sodium acetate pH 5.8 containing 2% sodium lauryl sulphate rather than manoxol.

Hydroxyproline assay. The concentration of guinea-pig skin salt soluble collagen solutions was determined by hydroxyproline estimation after the method of Woessner [8] with minor modifications.

Preparation of ^{35}S-proteoglycan substrate. Polyacrylamide beads containing entrapped ^{35}S-labelled proteoglycan molecules were prepared as described by Dingle *et al.* [9]. Normally 10–12 g of beads were obtained by this procedure having a specific activity of 5000–10,000 dpm/mg proteoglycan. ^{35}S-labelled proteoglycan prepared prior to incorporation in polyacrylamide beads was used as substrate in the Sepharose chromatography experiments.

Preparation of ^{14}C-labelled collagen. Neutral salt-soluble ^{14}C-glycine labelled guinea-pig skin collagen was prepared after the method of Gross [10]. Collagen solutions were prepared by dissolution of lyophilised collagen in potassium phosphate buffer (0.4 ionic strength) pH 7.6 at 2° and subsequent dialysis against 0.4 M NaCl at 2°. The collagen solutions normally had a specific activity in the range 15,000–20,000 dpm/mg collagen.

Collagenase assay. Culture media collagenase activity was assayed by measuring the release of soluble radioactive products from a pellet of thermally reconstituted ^{14}C-glycine labelled collagen fibrils by the method of Nagai *et al.* [11]. Twenty-five microliter aliquots of a ^{14}C-collagen solution in 0.5 ml capacity polypropylene tubes were allowed to gel at 37° for 24 hr. Incubations contained collagen gel, medium sample (usually 10–25 μl), and additives in 0.05 M Tris–HCl pH 7.6, 0.01 M $CaCl_2$, in a final volume of 0.2 ml. Tubes were incubated at 37° for 16 hr. The reaction was stopped by centrifugation at 10,000 *g* for 30 min and 0.1 ml aliquots of supernates were taken for scintillation counting. All assays were carried out in duplicate.

Proteoglycanase assay. Culture media were assayed for proteoglycanase activity as described by Dingle *et al.* [9]. Incubation tubes contained 0.5 ml ^{35}S-proteoglycan bead suspension (12.5 mg beads/ml H_2O), 0.25 ml media sample and additives in 0.1 M Tris–HCl pH 7.6, 0.01 M $CaCl_2$, in a final volume of 1 ml. Tubes were incubated at 37° for 16 hr. The reaction was stopped by the addition of 0.25 ml 0.5% (w/v) sodium lauryl sulphate in a 5% (v/v) solution of HCl. The tubes were centrifuged for 5 min at 1250 *g* to sediment the beads and 0.25 ml aliquots of the supernate were taken for scintillation counting. All samples were assayed in duplicate along with appropriate controls.

Determination of cartilage proteoglycan synthesis. Proteoglycan synthesis by articular cartilage explants was determined by uptake of $Na_2{}^{35}SO_4$ as follows. Cartilage slices (5 mm in length and 1 mm thick) in the presence or absence of synovial tissue and/or drugs were cultured in DMEM/5% rabbit serum as described previously. At stated times during culture 1 μCi $Na_2{}^{35}SO_4$ was added to appropriate cultures. After the incubation period (24 hr) media and synovial tissue were discarded (preliminary experiments had indicated that synovial tissue incorporated negligible amounts of isotope). Cartilage slices were washed 3 times with 1 ml vol of ice-cold 0.9% saline/1 mg/ml sodium sulphate to remove unbound label, and digested in 1 ml papain (0.1 U) in 0.1 M sodium acetate/acetic acid pH 5.7, 5 mM cysteine, 1 mM EDTA overnight at 37°. Samples of digests were taken for scintillation counting and chondroitin sulphate assays.

Determination of cartilage proteoglycan breakdown. To obtain ^{35}S-labelled cartilage proteoglycan, cartilage slices (5 mm in length and 1 mm thick) were incubated for 48 hr at 37° in 5 ml DMEM/5% rabbit serum containing 500 μCi $Na_2{}^{35}SO_4$ in a 95% air/5% CO_2 gas phase. Slices were then washed 10 times with 5 ml vol of DMEM/1 mg/ml sodium sulphate, whence radioactivity in the washing was reduced to background levels. In preliminary experiments the extent of isotope incorporation into proteoglycan aggregates was determined on papain digests of washed cartilage slices as follows. To 0.5 ml aliquots of the digests was added 0.05 ml cetyl pyridinium chloride (CPC) and the samples stood at room temperature for 30 min. After centrifugation at 3000 *g* for 5 min the supernates were removed and the pellets were solubilized by the addition of 0.25 ml formic acid and heating at 70° for 10 min. Formic acid (0.1 ml) was also added to the supernates. Aliquots of both supernates and pellet digests were counted for radioactivity. Results showed that at least 90% of label was precipitated with CPC indicating that most of the isotope had been incorporated into proteoglycan aggregates.

Labelled cartilage slices were then incubated alone or together with synovium or drugs as described previously for the required time intervals (see appropriate figures). After culture, the cartilage slices were washed with ice-cold saline/sodium sulphate solutions to remove unbound label and digested with papain as described previously. Samples of digests and corresponding culture media were taken for scintillation counting.

Scintillation counting. All samples were added to 10 ml scintillant (NE 260, Nuclear Enterprises) and counted in an LS 230 Beckman Scintillation counter.

RESULTS

The kinetics of synovium-induced cartilage matrix breakdown in vitro. When explants of living pig articular cartilage overlaid with a minced preparation of living synovial tissue from the same joint, were cultured for up to 14 days in DMEM supplemented with 5% heat-inactivated, acid denatured rabbit serum, the cartilage became depleted firstly of proteoglycan and subsequently collagen with resultant loss of tissue architecture. As shown in Fig. 1(a) proteoglycan loss had occurred in areas of cartilage overlaid by synovium by day 5. At this time there were no signs of cartilage collagen breakdown (Fig. 1b). After 14 days, however, loss of proteoglycan metachromasia had spread to all areas of the cartilage and collagen degradation was observed in regions of cartilage overlaid by synovial tissue (Fig. 1c).

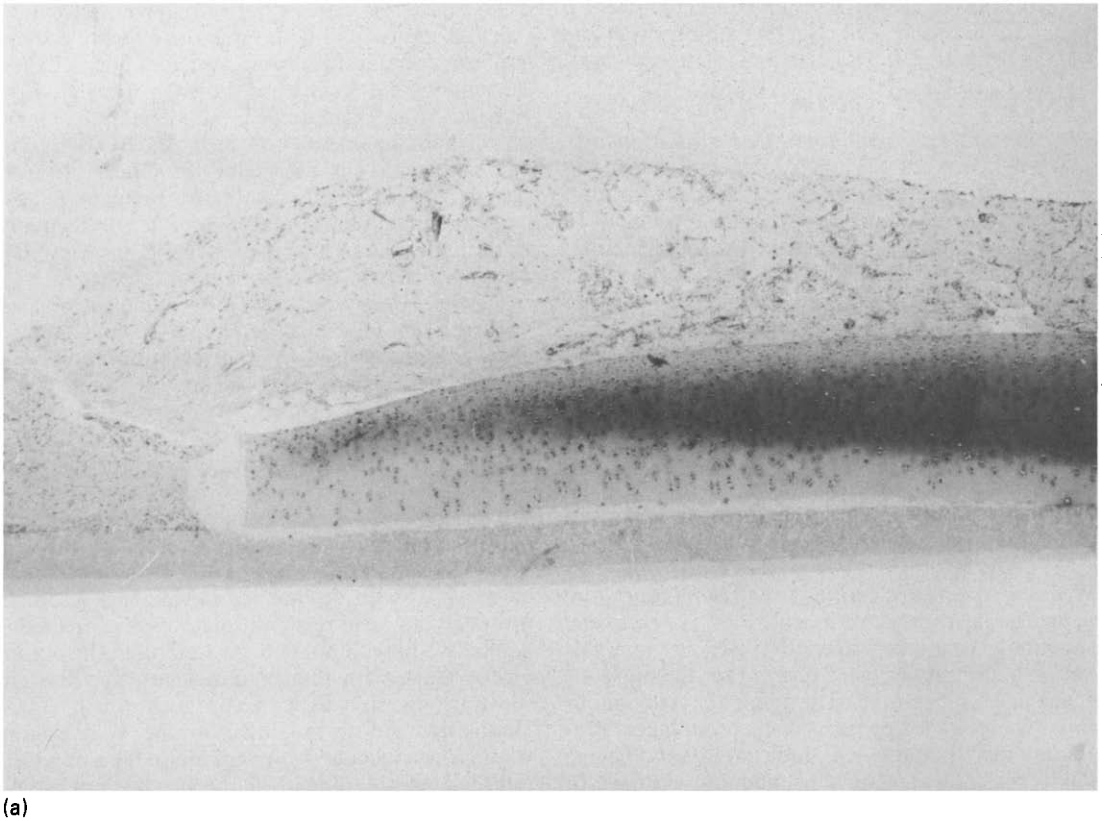
The detection of proteoglycan- and collagen-degrading enzymes in cartilage and synovium culture media. Media from cartilage, synovium and cartilage/synovium co-cultures collected at 2–3 day intervals over a 14 day period, were assayed for the presence of enzymes capable of degrading the two major matrix components, proteoglycan and collagen. Preliminary experiments had indicated that enzyme activity was not detectable in media of cartilage or synovium cultures when tissues were cultured in the presence of undenatured rabbit serum, probably due to released enzymes being in the form of enzyme-inhibitor complexes. Accordingly tissues were cultured in media supplemented with acid denatured serum (in which the α_2-macroglobulin is denatured). Under these conditions the kinetics of cartilage matrix breakdown were similar to those using undenatured serum. Both neutral proteoglycanase and collagenase activities were detected in the culture media and data from a typical experiment are shown in Fig. 2. Enzyme activity could only be detected in media from synovium-containing cultures indicating that both enzyme activities derived from this tissue. Treatment of media with either trypsin (20 μg/ml medium, 30 min, 30°) or 4-aminophenylmercuric acetate (0.7 mM) resulted in increased activity of both enzymes indicating that a proportion (up to 50% in some experiments) of both enzymes was released in latent form. Activation of medium samples, however, did not change the profile of enzyme activity over the culture period and accordingly media samples were not activated prior to assay in the experiments shown. Enzyme activity was not detectable in media from isolated cartilage cultures even after activation. The time course and degree of enzyme release showed interexperimental variations, but in six similar experiments peak neutral proteoglycanase levels occurred during the first week of culture whereas medium collagenase levels were highest during the second week. The appearance of both enzymes in the medium was abolished when tissues were cultured in media containing 1 μg/ml cycloheximide (Fig. 2) indicating that both enzymes were synthesized *de novo* during culture.

Effects of inhibitors on neutral proteoglycanase and collagenase. An active pool of pig synovial culture medium was used to determine the effect of enzyme inhibitors on neutral proteoglycanase and collagenase activity and results are shown in Table 1. Both enzymes were inhibited by undenatured bovine serum and the metal ion chelators EGTA, 1,10-phenanthroline and L-cysteine. Inhibitors of thiol-, serine- and carboxyl-proteinases (leupeptin, soya bean trysin inhibitor and pepstatin, respectively) were ineffective. These data support the view that both enzymes are neutral metalloproteinases.

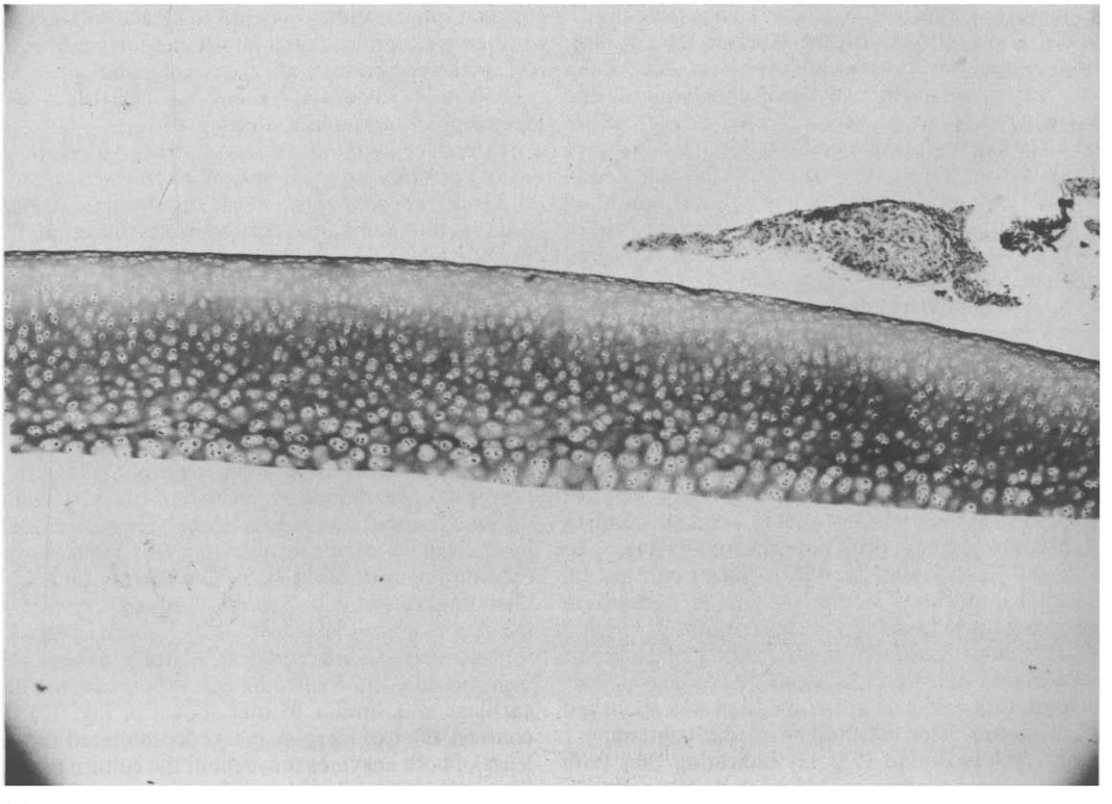
Further characterisation of pig synovial neutral proteoglycanase. The same active pool of pig synovial culture medium used for the inhibitor studies was used to further characterise the neutral proteoglycanase. When assayed over the pH range 3–9, a peak of proteoglycanase activity was observed at pH 7.5 (Fig. 3). Optimal collagenase activity was in the pH range 7–8.

To characterise the specificity of action of the neutral proteoglycanase against isolated cartilage proteoglycan, a sample of synovial medium was incubated at 37° with ^{35}S-labelled bovine nasal cartilage proteoglycan. The resultant digest was subjected to Sepharose-6B column chromatography. The elution profile along with that of unreacted substrate and substrate digested with papain is shown in Fig. 4. Undigested substrate eluted in the void volume whereas products of proteoglycanase digestion were retarded, eluting ahead of the products of papain digestion. The close correlation between the elution profiles of the proteoglycanase and papain digests is consistent with the view that synovial neutral proteoglycanase cleaves the core protein with the liberation of chondroitin sulphate peptides. The same crude enzyme preparation was able to cleave salt-soluble guinea-pig skin collagen molecules in solution at 26° with the production of characteristic 3/4 to 1/4 collagenase digestion products (Crossley and Hunneyball, unpublished observations).

Effects of anti-inflammatory drugs on cartilage matrix metabolism and synovial proteoglycanase and collagenase production. The effects of some currently used anti-inflammatory drugs on cartilage matrix breakdown and synovial proteoglycanase and collagenase activity was investigated for two reasons. Firstly, modification of enzyme yields and matrix breakdown by drugs may help to ascribe a role for these enzymes in this *in vitro* system and secondly such drug effects *in vitro* may help to highlight the actions of these compounds on cartilage breakdown *in vivo*. Figure 5 shows the effects of two non-steroidal anti-inflammatory drugs (NSAID), flurbiprofen and indomethacin, and two steroids, dexamethasone and prednisolone, on medium enzyme levels in cartilage/synovium cocultures during a 13-day period. Both NSAID's slightly increased medium levels of both enzymes but had no effect on the extent or progression of cartilage matrix degradation compared with non-drug cultures as assessed histologically (i.e. histological appearance of the cartilage was similar to that shown in Fig. 1c). In contrast the two steroids markedly inhibited the release of both enzymes throughout the culture period. These drugs inhibited totally the breakdown of cartilage collagen as judged histologically. However,



(a)



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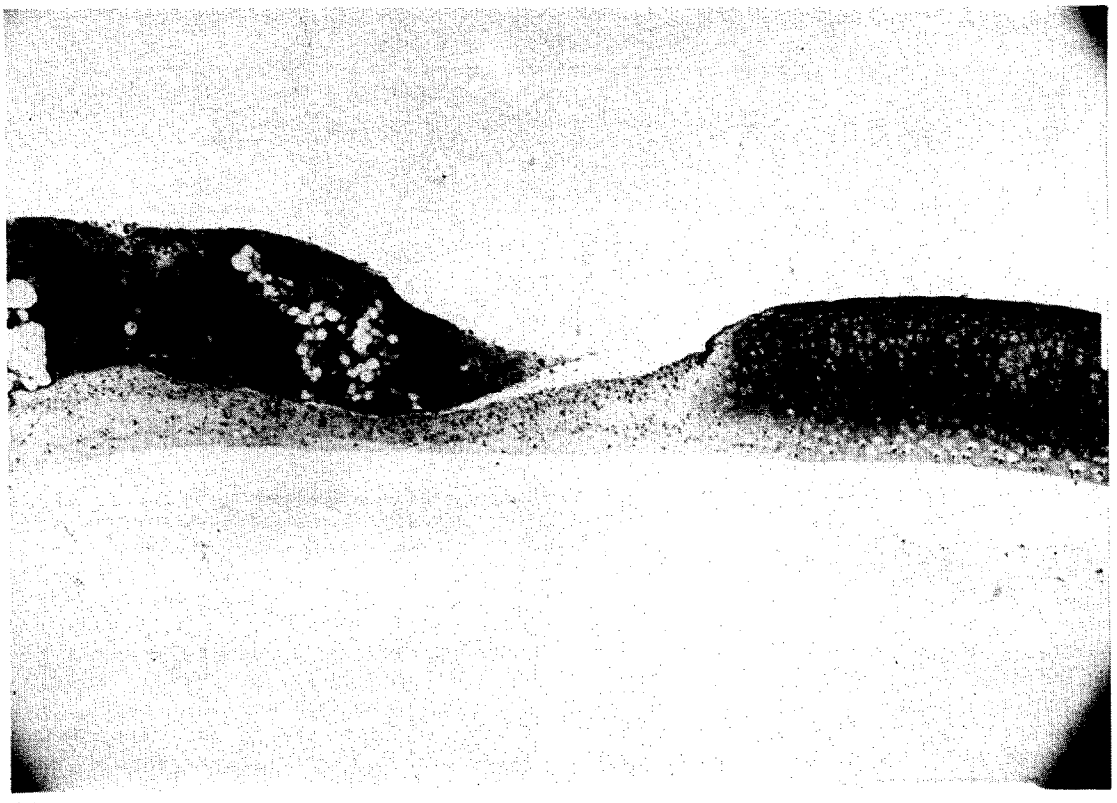

(c)

Fig. 1. Histology of cartilage/synovium at days 5 and 14 of culture. (a) Day 5, *o*-toluidine blue, ×50; (b) day 5; Van Gieson's, ×50; (c) day 14, Van Gieson's, ×50.

although the steroids markedly reduced medium proteoglycanase levels, synovium-induced cartilage proteoglycan breakdown was not inhibited as judged histologically.

To further investigate the effects of one steroid, dexamethasone, on proteoglycan metabolism, synthesis and breakdown were determined independently in the presence and absence of synovium. To do this synthesis was assessed by the uptake of $Na_2{}^{35}SO_4$ into newly-formed proteoglycan, and breakdown measured by the release of proteoglycan from cartilage slices pre-labelled *in vitro* with $Na_2{}^{35}SO_4$. The effects of dexamethasone and synovium on proteoglycan synthesis and breakdown were

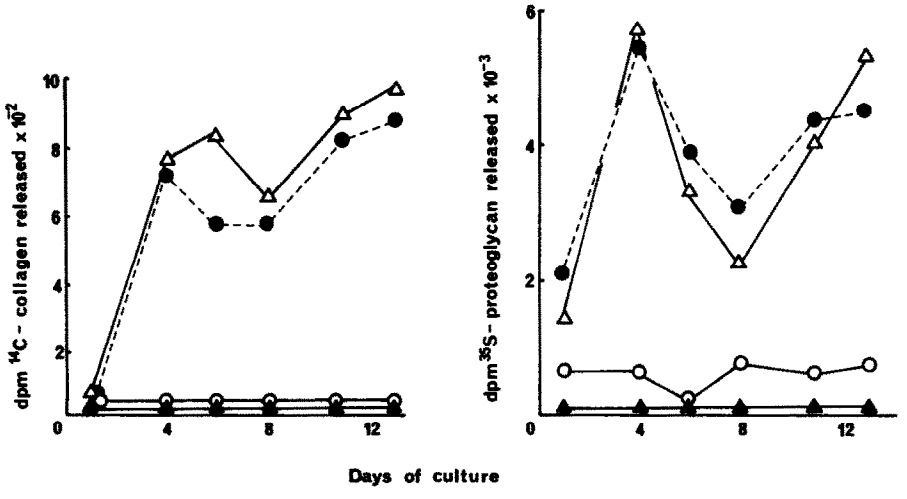

Fig. 2. Neutral proteoglycanase and collagenase levels in pig cartilage and synovium culture media and the effect of cycloheximide. Neutral proteoglycanase and collagenase activities at pH 7.6 were determined in media of cartilage (○——○), synovium (△——△), cartilage/synovium co-cultures (●——●), and cartilage/synovium cultured in the presence of cycloheximide (1 μg/ml) (▲——▲).

Table 1. Effect of inhibitors on pig synovial neutral proteoglycanase and collagenase activity

Inhibitor	Concentration in assay	Inhibition of enzyme activity %	
		Neutral proteoglycanase	Collagenase
None	—	0	0
Bovine serum	10% v/v	98.0	92.0
EGTA	10 mM	93.2	100.0
1,10-Phenanthroline	5 mM	99.6	99.2
L-Cysteine	10 mM	94.0	97.0
Leupeptin	25 μg/ml	12.5	0
SBTI	1 mg	17.0	0
Pepstatin	75 μ/ml	0	0

Abbreviations: SBTI, soya bean trypsin inhibitor; EGTA, ethyleneglycol-bis-(β-amino ethyl ether) *N,N'*-tetra acetic acid.

The effect of inhibitors on the neutral proteoglycanase and collagenase activity was determined using the same preparation of pig synovial culture medium. Inhibitors were preincubated with medium samples for 20 min at 25° prior to assay. All assays were carried out in duplicate and incubations were for 16 hr at 37°.

investigated during the first 7–8 days of culture, i.e. the period in which maximal synovium-mediated proteoglycan depletion occurs. Isolated cartilage explants lost up to one third of their proteoglycan content during the first week of culture, the rate of loss being maximal during the first 4 days (Fig. 6). Neither the rate nor extent of proteoglycan depletion was affected in cartilage cultures containing dexamethasone (10^{-6} M). Proteoglycan breakdown was increased in cartilage/synovium co-cultures and this synovium-mediated degradation was partially inhibited by the steroid.

The effects of dexamethasone on cartilage proteoglycan synthesis in the presence and absence of synovium were investigated and results from a typical experiment are shown in Fig. 7. As preliminary experiments had indicated that the rate of loss of unlabelled proteoglycan from cartilage or cartilage/synovium cultures was similar to that of labelled material, synthesis was expressed in terms of specific activities, i.e. dpm/μg cartilage chondroitin sulphate. Both the steroid and synovial tissue severely reduced cartilage proteoglycan synthesis during the first week of culture. Interestingly, in similar experiments it was a common observation that dexamethasone did not inhibit proteoglycan synthesis during the first 48 hr of culture, whereas an inhibition by synovium occurred from the outset. In other similar experiments neither flurbiprofen nor indomethacin, both at 10^{-5} M, had any effect on cartilage proteoglycan synthesis or degradation in the absence or presence of synovium.

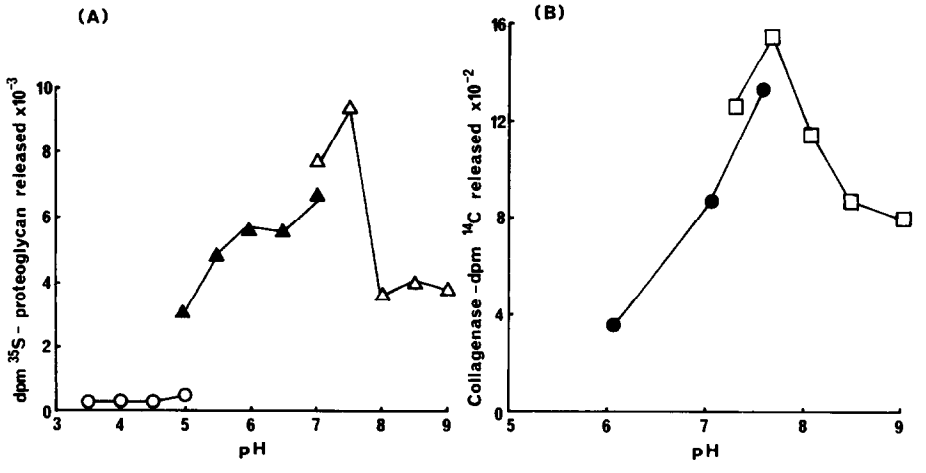

Fig. 3. Proteoglycanase and collagenase activity of pig synovial culture media at different pH values. Proteoglycanase (A) and collagenase (B) levels in pig synovial culture media are shown. Incubations were for 16 hr at 37° and buffers used were for proteoglycanase: 0.1 M sodium acetate/acetic acid pH 3.5–5.0 (○——○), 0.1 M sodium cacodylate/HCl pH 5.0–7.0 (▲——▲), and 0.1 M Tris–HCl, pH 7.0–9.0 (△——△) and for collagenase; 50 mM Tris–maleate/NaOH, pH 6.0–7.6 (●——●) and 50 mM Tris–HCl, pH 7.3–9.0 (□——□).

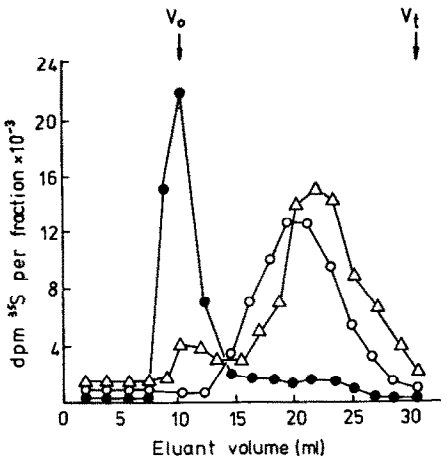

Fig. 4. Sepharose chromatography of ^{35}S-proteoglycan after digestion with pig synovial culture medium or papain. Twenty microliters of a solution of ^{35}S-labelled bovine nasal cartilage proteoglycan (30 mg proteoglycan/ml) was incubated for 16 hr at 37° with either pig synovial culture medium (0.5 ml) in 0.1 M Tris–HCl buffer, pH 7.6, containing 10 mM $CaCl_2$ (final vol. 1.0 ml) or papain (0.1 U) in 0.1 M sodium acetate/acetic acid buffer, pH 5.7, containing 5 mM cysteine, 1 mM EDTA (final vol. 1 ml). Digests (0.5 ml) along with unreacted substrate were chromatographed on a column (25 × 1.5 cm) of Sepharose-6B equilibrated with 0.05 M sodium cacodylate/HCl buffer pH 7.0 containing 0.5 M NaCl, 0.2 mg/ml sodium azide which also served as the eluting buffer. Fractions (1.8 ml) eluted at a flow rate of 30 ml/hr at room temperature were collected and 0.1 ml aliquots taken for scintillation counting. Elution profiles shown are to unreacted substrate (●——●), and substrate digested with either pig synovial culture medium (○——○) or papain (△——△). Blue dextran and $Na_2{}^{35}SO_4$ were used to determine V_o and V_t respectively.

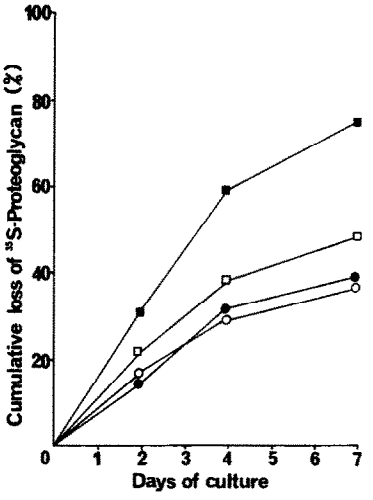

Fig. 6. Effect of dexamethasone and synovium on pig cartilage proteoglycan breakdown *in vitro*. ^{35}S-labelled cartilage explants were cultured alone (○——○) or in the presence of dexamethasone (10^{-6} M) (●——●), synovial tissue (■——■), or both (□——□).

The percentage loss of ^{35}S-proteoglycan was calculated from the formula:

$$\frac{\text{dpm } ^{35}S \text{ in culture medium}}{\text{dpm } ^{35}S \text{ in culture medium} + \text{dpm } ^{35}S \text{ in cartilage digests}} \times 100.$$

Each value is the mean of pooled samples from four replicate cultures.

DISCUSSION

Data have been presented which illustrate that slices of pig articular cartilage, when overlaid with a minced preparation of synovium from the same joint,

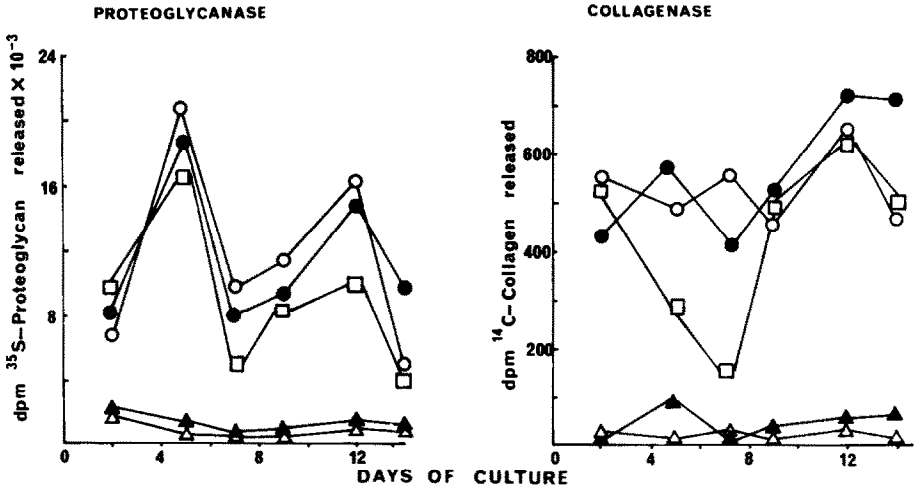

Fig. 5. Effect of steroidal and non-steroidal anti-inflammatory drugs (NSAID) on medium levels of neutral proteoglycanase and collogenase from pig cartilage/synovium co-cultures. Results shown are from cultures without drug (□——□) and from cultures containing 10^{-5} M flurbiprofen (●——●); 10^{-5} M indomethacin (○——○); 10^{-6} M prednisolone (▲——▲) and 10^{-6} M dexamethasone (△——△). Each data point is the mean value of pooled medium from four separate cultures.

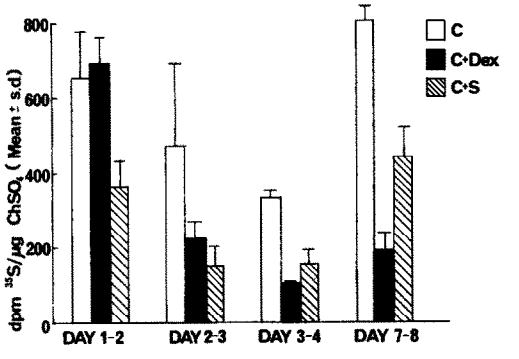

Fig. 7. Effect of pig synovium and dexamethasone on cartilage proteoglycan synthesis *in vitro*. Cartilage proteoglycan synthesis by isolated explants (C) or explants cultured with synovial tissue (C + S) or dexamethasone (10^{-6} M) (C + Dex) was determined by uptake of $Na_2{}^{35}SO_4$. Rates of synthesis were assessed during the time intervals shown and each value represents the mean ± S.D. of four replicate cultures.

undergo extensive proteoglycan and collagen breakdown during a 2-week culture period. The sequential depletion of cartilage matrix components was manifest firstly as a net loss of proteoglycan (during the first week of culture) and subsequently as collagen breakdown in areas of cartilage denuded of proteoglycan (particularly under the leading edge of the synovium). These observations accord with those of Fell and Jubb [2] who concluded that synovium-induced matrix degradation occurred by two distinct mechanisms; one, a direct attack by presumably synovial enzymes which required the two tissues to be in contact, and the other through release of a soluble factor from synovial cells which stimulated chondrocytes to degrade their own matrix. This latter effect required living chondrocytes.

We have shown that under culture conditions where serum proteinase inhibitors are denatured the synovial tissue synthesises and releases collagen- and proteoglycan-degrading enzymes. The collagenase had properties similar to that purified and characterised from pig synovial tissue [12, 13]. Using Sepharose-6B chromatography we have shown that the neutral proteoglycanase attacks isolated proteoglycan with the liberation of products slightly larger than those produced by papain digestion, a proteinase which degrades cartilage proteoglycan with the formation of peptide fragments containing single glycosaminoglycan chains [14]. Partial characterisation of this putative enzyme showed it to have a similar pH optimum and inhibitory properties to that of the collagenase. We did not attempt to separate the enzyme activities by physical means and it is therefore possible that the activities directed against collagen and proteoglycan substrates are attributable to a single enzyme. It is of interest, however, that the collagenase and proteoglycanase isolated from the culture media of mouse bone explants could not be satisfactorily separated by physical means [15].

The effects of some anti-inflammatory drugs on synovium-induced cartilage breakdown were examined in order to ascertain whether these agents may modulate cartilage metabolism via an effect on synovial enzymes. Such drug investigations are not without precedent. The breakdown of normal rabbit articular cartilage proteoglycan by arthritic rabbit synovium was inhibited by both hydrocortisone and indomethacin [16], whereas hydrocortisone but not indomethacin inhibited the release of bovine nasal cartilage proteoglycan stimulated by human rheumatoid synovium [17]. Dexamethasone, flufenamic acid, chloroquine and aurothiomalate inhibited the production of a neutral proteoglycanase by thioglycollate-stimulated mouse peritoneal macrophages [18] and dexamethasone and hydrocortisone inhibited the appearance of collagenase in the media of normal human skin explants [19]. In addition, dexamethasone and hydrocortisone inhibited the appearance of collagenase in the media of cultured explants of human rheumatoid and rabbit arthritic synovium via a suppressive effect on collagenase synthesis allied to an increased production of a tissue inhibitor of metalloproteinases [20, 21].

In our studies the NSAID's, flurbiprofen and indomethacin had little or no effect on synovial enzyme production or the kinetics of synovium-induced cartilage degradation. These observations may relate to the lack of effect of this class of drug on the disease progress in human rheumatoid arthritis [22] and in the experimental model of arthritis in rabbits [23]. In contrast, the steroids dexamethasone and prednisolone inhibited the synthesis of both synovial enzymes. Although cartilage collagen breakdown was inhibited by the steroids, cartilage proteoglycan breakdown was largely unimpaired as judged histologically. In order to interpret the lack of correlation between proteoglycanase inhibition and proteoglycan breakdown it seemed relevant to examine the effects of steroids on both cartilage proteoglycan synthesis and breakdown. Both dexamethasone and synovial tissue inhibited cartilage proteoglycan synthesis. The inhibitory effect of pig synovium on cartil age proteoglycan synthesis has been reported previously [24] and Lippiello *et al.* [25] found that short term incubations of rabbit articular cartilage in media from cultures of human rheumatoid synovium resulted in marked inhibition of $^{35}SO_4$ incorporation into the cartilage matrix. These *in vitro* observations correlated with the *in vivo* demonstration of a reduction in $^{35}SO_4$ uptake by sections of cartilage from the joints of rabbits and mice with experimental antigen-induced arthritis [26, 27]. Also Behrens *et al.* [28] showed that intra-articular injections of hydrocortisone into rabbit knees inhibited proteoglycan synthesis by rabbit articular cartilage. From studies using prelabelled cartilage slices we have shown that isolated explants may lose up to a third of their proteoglycan content during eight days of culture, this loss being greatest during the first four days; dexamethasone had no effect on this process. Synovial tissue markedly increased proteoglycan depletion and this increase was partially inhibited by dexamethasone. In similar studies Tyler *et al.* [29] showed that hydrocortisone suppressed synovium-mediated cartilage proteoglycan depletion via an effect on the degradative process whereas the drug did not inhibit proteoglycan synthesis by isolated cartilage explants assessed histologically. From our studies, to date,

one explanation for the lack of correlation between inhibition of synovial proteoglycanase production by steroids and quantitative observations on cartilage proteoglycan depletion would be that the inhibitory effect of both the steroid and synovial tissue on proteoglycan synthesis results in net proteoglycan loss. Alternatively, the effects of synovial tissue on cartilage proteoglycan breakdown may be mediated, in part, by a soluble synovial factor(s), catabolin, which stimulates living chondrocytes to degrade their own matrix. In this respect it is interesting that pig synovial catabolin production *in vitro* can be inhibited by prednisolone [30]. In the first instance, the relative contributions of synovial proteoglycanase and catabolin could be investigated in studies using dead cartilage where the effects of catabolin would be nullified. However, this would make the culture system less relevant to the situation *in vivo*. More conclusive evidence may be obtained by the use of specific antisera to both purified enzymes and catabolic factors.

Inhibition of cartilage collagen breakdown by steroids is consistent with inhibition of synovial collagenase production. Evidence presented by Jubb [31] suggests that cartilage collagen breakdown may be mediated largely by synovial collagenase; under conditions of hyperoxia an increase in pig cartilage collagen breakdown by homologous synovial tissue correlated with an increase in synovial collagenase production whereas synovial catabolin production was inhibited. If this is the case then the inhibition of collagenase production by steroids may represent a mechanism for the inhibition of erosive progression when these drugs are used in high doses in patients with rheumatoid arthritis [32].

In this paper we have described the production of proteolytic enzymes during the co-culture of pig synovium and cartilage *in vitro* and the effects of selected anti-inflammatory drugs thereon. The inhibitory effects of the steroids on the production of the proteolytic enzymes may, in part, explain the effects of these drugs in inhibiting the breakdown of cartilage collagen. However, cartilage matrix protein breakdown appears to occur via more than one mechanism and the inhibitory action of steroids on catabolin production may be more important. To investigate further the relative importance of drug effects on these two mechanisms of breakdown may not be easy since both catabolin and synovial enzymes may be acting in an interdependent manner. Nevertheless further studies with this type of culture system should help to elucidate the mechanism of action of corticosteroids in inflammatory joint diseases.

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REFERENCES

1. S. M. Krane, *Ann. rheum. Dis.* **40**, 433 (1981).
2. H. B. Fell and R. W. Jubb, *Arthritis Rheum.* **20**, 1359 (1977).
3. J. T. Dingle, J. Saklatvala, R. Hembry, J. Tyler, H. B. Fell and R. W. Jubb, *Biochem. J.* **184**, 177 (1979).
4. J. Saklatvala, *Biochem. J.* **199**, 705 (1981).
5. Z. Werb and S. Gordon, *J. exp. Med.* **142**, 346 (1975).
6. J. C. Unkeless, S. Gordon and E. Reich, *J. exp. Med.* **139**, 834 (1974).
7. P. Whiteman, *Biochem. J.* **131**, 343 (1973).
8. J. F. Woessner Jr., *Archs. Biochem. Biophys.* **93**, 440 (1961).
9. J. T. Dingle, A. M. J. Blow, A. J. Barrett and P. E. N. Martin, *Biochem. J.* **167**, 775 (1977).
10. J. Gross, *J. exp. Med.* **107**, 247 (1958).
11. Y. Nagai, C. M. Lapiere and J. Gross, *Biochemistry* **5**, 3123 (1966).
12. T. E. Cawston and J. A. Tyler, *Biochem. J.* **183**, 647 (1979).
13. J. A. Tyler and T. E. Cawston, *Biochem. J.* **189**, 349 (1980).
14. R. W. Stephens, L. I. Pemberton, P. Ghosh and T. K. Taylor, *Analyt. Biochem.* **90**, 726 (1978).
15. G. Vaes, Y. Eeckhout, G. Lenaers-Claeys, C. Francois-Gillet and J. E. Druetz, *Biochem. J.* **172**, 261 (1978).
16. J. Panagides, M. J. Landes and A. E. Sloboda, *Agents Actions* **10**, 22 (1980).
17. J. Steinberg, S. Tsukamoto and C. B. Sledge, *Arthritis Rheum.* **22**, 877 (1979).
18. N. R. Ackerman, S. N. Jubb and S. L. Marlowe, *Biochem. Pharmac.* **30**, 2147 (1981).
19. T. J. Koob, J. J. Jeffrey and A. Z. Eisen, *Biochem. biophys. Res. Commun.* **61**, 1083 (1974).
20. M. B. McGuire, G. Murphy, J. J. Reynolds and R. G. G. Russell, *Clin. Sci.* **61**, 703 (1981).
21. G. J. Cambray, G. Murphy and J. J. Reynolds, *Rheum. Int.* **1**, 69 (1981).
22. V. Wright and R. Amos, *Br. med. J.* **280**, 964 (1980).
23. M. B. Goldlust and L. C. Rich, *Agents Actions* **11**, 730 (1981).
24. R. W. Jubb and H. B. Fell, *Arthritis Rheum.* **23**, 545 (1980).
25. L. Lippiello, K. Yamamoto, D. Robinson and H. J. Mankin, *Arthritis Rheum.* **21**, 909 (1978).
26. J. D. Sandy, D. A. Lowther, and H. L. G. Brown, *Arthritis Rheum.* **23**, 433 (1980).
27. W. B. van Den Berg, M. W. M. Kruijsen, L. B. A. van de Putte, H. J. van Beusekom, M. van der Sluis-van der Pol and W. A. Zwarts, *Br. J. exp. Path.* **62**, 308 (1981).
28. F. Behrens, N. Shepard and N. Mitchell, *J. Bone Jt. Surg. Am.* **58-A**, 1157 (1976).
29. J. A. Tyler, H. B. Fell and C. E. Lawrence, *J. Path.* **137**, 335 (1982).
30. H. Sheppeard, L. M. C. Pilsworth, B. Hazleman and J. T. Dingle, *Ann. rheum. Dis.* **40**, 202 (1981).
31. R. W. Jubb, *Ann. rheum. Dis.* **38**, 279 (1979).
32. Joint Committee of the Medical Research Council and the Nuffield Foundation of Clinical Trials of Cortisone, ACTH and other Therapeutic Measures in Chronic Rheumatic Diseases, *Ann. rheum. Dis.* **18**, 173 (1959).