Extrusion of Pyrophosphate into Extracellular Media by Osteoarthritic Cartilage Incubates

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ABSTRACT The distribution of calcium pyrophosphate mineral phase, almost exclusively confined to articular cartilage in chondrocalcinosis, and the high level of pyrophosphate (PPi) ion relative to serum in synovial fluid in patients with either chondrocalcinosis or advanced osteoarthritis led to an investigation of whether cartilage cells elaborate PPi ions.

Incubates of articular cartilage from young rabbits but not from mature rabbits, as well as growth plates cartilage, released PPi into incubation media during a 4-h period. Control rabbit ear cartilage and synovial membrane elaborated negligible amounts of PPi. The PPi was shown to be undialyzable but could be dissociated from the alkaline phosphatase by ultracentrifugation. In 16 patients with osteoarthritis, a substantial output of PPi by samples of articular cartilage from the knee was demonstrated. It is postulated that either rapid cell division and matrix synthesis found in the base of ulcerating osteoarthritic cartilage or remodeling calcified sites are the source of the PPi in such osteoarthritic cartilage. It is further hypothesized that this PPi output accounts at least in part for the elevated PPi levels found in synovial fluid of patients with osteoarthritis.

INTRODUCTION

Clinical features of chondrocalcinosis during the last decade have attracted considerable attention (1-8), but information concerning fundamental biochemical defects that would explain the deposition of the mineral phase, calcium pyrophosphate dihydrate (CaPPi) (9), found in articular cartilage, is meager. The major biochemical abnormality found so far has been a substantial synovial fluid-plasma inorganic pyrophosphate gradient (10-12); in one study red cell neutral Mg-dependent pyrophosphatase in a group of patients with chondrocalcinosis was not reduced below levels found in normal control subjects (13), and despite some contrary evidence (10, 14, 15), synovial fluid alkaline phosphatase levels have in most cases been within normal limits. McCarty has proposed, partly because of the above-stated data and a host of negative systemic abnormalities, as well as histological features of chondrocalcinosis, that the articular cartilage is the most likely site of initial mineral deposition (11). This theory implicates some error in local cartilage metabolism that specifically predisposes it to develop CaPPi deposition, even when hydroxyapatite in the same person is deposited ectopically in the aorta or other nonarticular sites (9).

In our previous report a high concentration ratio of PPi synovial fluid to plasma was found in patients with chondrocalcinosis, as expected from aforementioned studies (10-12), but surprisingly, a similar finding was present in osteoarthritis patients (12), a result confirmed by Silcox and McCarty (16).

An explanation of this phenomenon might bring insight concerning peculiarities of cartilage metabolism relating to chondrocalcinosis and the characteristic deposition of CaPPi. In particular, elucidation of PPi handling by normal and osteoarthritic cartilage seemed a logical point from which to begin, because of the much greater opportunity for sampling human osteoarthritic than chondrocalcinotic cartilage.

A severe restriction on the study of cartilage metabolism of PPi in animals or man has been the lack of methods to analyze PPi in the minute concentrations normally present in body fluids or released into incubation media for in vitro tissue studies. In the present report a miniaturized PPi analytical method, published before in preliminary form (12), is described here in detail, and during initial experiments data on incubates of rabbit cartilage have provided evidence for surprising

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elaboration of unhydrolyzed PPi from proliferating cartilages. In addition, fresh cartilage from a well-characterized series of osteoarthritic patients revealed consistent, small elaboration of PPi into incubation media in comparison to controls.

METHODS

Animals. 1–6-mo-old and 2-yr-old normal female New Zealand white rabbits were killed with intravenous 100 mg/kg Nembutal (Abbott Laboratories, North Chicago, Ill.), and the tissues were handled as described below. Body weight for these rabbits ranged from 0.3 to 2.3 kg and from 4.2 to 4.7 kg, respectively.

Patients. All patients gave a complete medical history and received a physical examination during the years 1972–74 at Jackson Memorial Hospital and Miami Veterans Administration Hospital; 16 patients were judged to have primary osteoarthritis based on exclusion of other diseases. Evaluation of knee and hip x-rays by the criteria of Kellgren and Lawrence (17) on each patient revealed moderate to severe disease (grades 2 to 4). Cartilage was, in all cases, obtained at orthopedic surgery for total knee or hip replacement. Control cartilage was obtained from hips of patients who had suffered femoral neck fractures and from two patients at the time of above-knee amputation ("normal," Table IV). Any patients receiving recent intra-articular injections of corticosteroids, etc., were excluded.

Tissue preparations and incubations. Cartilages were promptly dissected fresh and incubated within 10 min of removal after processing as described below. All tissue samples were taken from margins of ulcerated cartilage in a weight-bearing site. Portions of each sample were fixed, embedded, cut, and stained with safranin O, as well as hematoxylin and eosin. Histological examination of a portion of each incubated cartilage revealed cell cloning and deep fissures (grades 5 to 12) by histological criteria of Mankin et al. (18), particularly in the radial and transitional zones. No evidence of chondrocalcinosis was found upon histological preparations subjected to conventional von Kossa stains. No evidence of calcium pyrophosphate crystals was seen in joint fluid, synovium, or articular cartilage, grossly or microscopically, although only conventional stains were employed.

In the rabbits, the cartilage covering the distal end of the femur and the proximal end of the tibia were resected. Four to five slices of cartilage were resected from each joint, with a sharp scalpel. The slices were diced in small pieces, approximately 0.3–0.5 mm thick. All samples were next placed in cool 0.9% saline solution.

In the same manner samples prepared from the rabbit ear cartilage freed of perichondrium and synovial membrane extirpated from the knee joints were washed three times with basal Eagle medium. In other experiments, the articular cartilage and growth plates of 1–6-mo-old rabbits were exposed by a sagittal incision under a dissecting microscope at 100 × magnification. The growth cartilage was divided into two cross-sectional pieces, containing (*a*) principally resting cell cartilage and (*b*) proliferating cell zone and hypertrophic cell zone. Histological study of each sample was made as described previously (19) to verify a gross zonal separation. In the same experiments, articular cartilage was divided into two further layers (*c*) containing predominantly tangential and transitional zones, and (*d*) predominantly radial zones, also containing growth cartilage with proliferating and hypertrophic cells. In adult rabbits (age 2 yr) the articular cartilage, now lacking growth cartilage per se, was divided as in (*c*) and (*d*). These sample were cut into blocks and incubated as described above.

Thereafter the material (50–90 mg wet wt) obtained from one animal or human was equally distributed among six 1-ml beakers, each containing 300 μl of the incubating basal Eagle medium. 5% CO_2, 95% air with 85% humidity was circulated for 30 s in the upper part of the beaker through a pipette with a cotton plug, and the beakers were tightly covered with parafilm (American Can Company, New York). Incubations were conducted at 38°C and maintained for 0, ½, 1, 2, 3, 4, and 6 h. The enzymic hydrolysis of PPi in the incubation was terminated by removal of the cartilage and addition of Cleland's reagent as described below. Solution pH was checked with microelectrodes as reported previously, and pH values ranged from 7.1 to 7.5 during the incubations. In each set of experiments on articular cartilage, control ear cartilage and knee synovial membrane from the same animal were incubated concurrently.

UDP glucose, NADP, UDP glucose pyrophosphorylase, colchicine, and actinomycin D were purchased from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol (Cleland's reagent), glucose 1–6 diphosphate, phosphoglucomutase, glucose 6-phosphate dehydrogenase, and *p*-nitrophenyl phosphate were purchased from Calbiochem, San Diego, Calif. [*methyl*-^{3}H]Thymidine was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. [5-^{3}H]cytidine was from New England Nuclear, Boston, Mass., and disodium ethane-1-hydroxy-1,1-diphosphate (EHDP) [1] was donated by Procter & Gamble Co., Cincinnati, Ohio.

Enzymatic determination of PPi. The enzymatic assay for PPi described in the current study was adapted from the three basic reactions described by Munch-Petersen (20):

$$\text{UDP} - \text{glucose} + \text{PPi} \leftrightharpoons \text{UTP} + \text{G-1-P} \quad (1)$$

$$\text{G-1-P} \leftrightharpoons \text{G-6-P} \quad (2)$$

$$\text{G-6-P} + \text{NADP} + \leftrightharpoons \text{gluconolactone-6-P} + \text{H}^+ + \text{NADPH} \quad (3)$$

Reaction 1 is catalysed by UDP-glucose pyrophosphorylase, reaction 2 by phosphoglucomutase, and reaction 3 by glucose-6-phosphate dehydrogenase. A similar adaptation has been published for the assay of RNA polymerase (21). PPi analyses were first made on standard PPi solutions prepared with the basal Eagle medium along with the unknowns. During testing of the method, samples of human plasma and synovial fluid were divided into two fractions immediately after being withdrawn; to both of them, Cleland's reagent was added to a final concentration of 10 mM. To only one of them 8 μM $Na_4P_2O_7$ was added as a recovery control. Both fractions were then centrifuged at 5°C for 15 min at 700 *g* and deproteinized by ultrafiltration at about 5°C, employing an Amicon PM-10 membrane (Amicon Corp., Lexington, Mass.) with the ultrafiltration cell of 10.0 ml capacity and a pressure of 45 lb/in^2 produced with a mixture of 5% CO_2 and 95% air. About 0.8 ml of the filtered sample was incorporated in the reaction mixture, which had a total volume of 1.10 ml and composition in addition as follows (final concentrations): Mg Cl_2, 5 mM; NADP, 0.6 mM; UDP-glucose 0.5 mM; Cleland's reagent, 10 mM; glucose 1–6 diphosphate, 50 nM; and 0.4 U

[1] *Abbreviation used in this paper:* EHDP, disodium ethane-1-hydroxy-1,1-diphosphonate.

of each of the following enzymes: glucose-6-phosphate dehydrogenase, phosphoglucomutase, and UDP glucose pyrophosphorylase. After addition of this last enzyme to the system, the reaction required about 15 min for completion at room temperature. The absorbance corresponding to the resulting NADPH was read in a 5.0-cm cuvette in a PMQ II Zeiss spectrophotometer (Carl Zeiss Inc., New York) at 340 nm wave length. A reagent blank was routinely run in each determination and consisted of the sample plus reaction mixture except for the first enzyme, UDP glucose pyrophosphorylase. When samples of ultrafiltered sample were incubated with yeast inorganic pyrophosphatase and then analyzed, the change in absorbance observed was equal to that of the blank, which confirms the specificity of the method of PPi.

The sensitivity of this method was tested with standard solutions. The theoretical absorbance for 1.0 nmol of NADPH at wave length of 340 nm in the 5-cm optical path cuvette is 0.031. The maximum spectrophotometer error, found at concentration level of 10 nmol, was ±1.0%, and at the 1.0 nmol level, ±6.5%. The PPi concentration found for human plasma with this method (12) ranged from 1.8 to 6.0 μM, consistent with values reported in the literature (10, 11).

For the present work, very small sample volumes were used; about 300 μl of the tissue culture solution medium was centrifuged in the Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 10,000 *g* for 5 min, and the supernate, to which Cleland's reagent was incorporated to a final concentration of 10 mM, was filtered through GSWP 01300, GS 0.22 μm; 13-mm diameter Millipore filter (Millipore Corp., Bedford, Mass.) in a XX30 01200 Swinny filter holder at 5°C and under 60 lb/in² pressure, obtained with a mixture of 95% air and 5% CO_2. Samples of 40 μl of the filtered solutions were incorporated in the reaction mixture, containing all of the aforementioned components at the concentrations indicated above, to a final volume of 55 μl. Immediately after being mixed, the system was transferred to a glass capillary tube holding 50 μl in 5-cm length. The capillary tube constituted a microcuvette, similar to that described in a prior publication (22), and the change in absorbance was registered.

In addition, two controls were run identically for each determination, with 40-μl aliquots of the same filtered solution. One was used as a blank after incubation with yeast inorganic pyrophosphatase. To the other, a known amount of 8 μM $Na_4P_2O_7$ was added as a recovery control. Addition of testicular hyaluronidase (Worthington Biochemical Corp., Freehold, N. J.) (1,500 U/ml for 30 min) concurrent with this filtration method did not increase the yield of PPi regardless of synovial or incubate fluid viscosity, so this step has not been included.

DNA determination. Samples of articular, growth plate, and ear cartilages as well as of synovial membrane after completion of the tissue incubations above were pooled, dehydrated, weighed, and homogenized with an all-glass, motor-driven dual tissue grinder (Kontes Glass Co., Vineland, N. J.) in 0.9% saline solution. The tubes were kept in ice water during grinding. The suspensions obtained were mixed vol/vol with 10% trichloroacetic acid (TCA), refrigerated for 30 min, and then centrifuged at 10,000 *g* for 5 min at 5°C. The precipitates were then washed twice with cold 5% TCA.

Extraction of DNA was next carried out with three successive additions of 5% TCA (about 0.3 ml each for 6 mg of dry tissue). After solvent addition, the system was heated at 90°C for 15 min, and the fluid was removed and replaced with fresh TCA. The combined extracts, as well as DNA standard solutions, were then mixed vol/vol with the color reagent (0.04% indole and hydrochloric acid sp gr 1.19, Mallinckrodt analytical reagents, vol/vol), and absorption was measured according to the method of Ceriotti (23). Experiments with standard DNA solutions were performed in parallel throughout the whole procedure.

Enzyme determinations. Alkaline phosphatase: To 50-μl aliquots of the incubating medium, 0.2 ml of the substrate mixture containing 0.1 M bicarbonate/carbonate buffer, pH 10.2, 10 mM *p*-nitrophenylphosphate, and 10 mM Mg Cl_2 was added. After a selected incubation period of 30–60 min at 38°C, the reaction was arrested by adding 0.9 ml 1 N NaOH solution containing 0.2 M EDTA. The resulting *p*-nitrophenol absorbance was measured at 400-nm wave length in a 5.0-cm optical path Zeiss cuvette.[2] Lactic dehydrogenase was measured by a conventional method (24).

Controls for cellular viability during incubation

[methyl-³H]Thymidine and [5-³H]cytidine incorporation. Samples of the aforementioned rabbit and human tissues were processed as described above and incubated for 4 h at 38°C in basal Eagle medium containing 13 μCi/beaker of either [*methyl* ³H]thymidine, 3 Ci/mM, or [5-³H]cytidine, 20 Ci/mM. The incorporated radioactivity was assayed by the method of Mankin and Lippiello (25), except for the following modifications: (*a*) the fixation in buffered 10% formaldehyde, pH 7.0, was for 2 hr; (*b*) the Celite filter aid was replaced by a drop of human serum; (*c*) filtration was substituted by centrifucation at 5°C at 700 *g* for 5 min *b* and *c* according to Hughes and Caspary (26).

Tissue autoradiography. A high resolution autoradiographic technique (27) provided qualitative evidence for the incorporation and distribution of labeled thymidine uptake by the cartilage. Tissue samples treated and incubated as described above for biochemical radioactivity incorporation were also fixed in formaldehyde, embedded in paraffin, cut into sections 5 μ thick and then coated with a thin layer of Kodak NTB_3 photograph emulsion (Eastman Kodak Co., Rochester, N. Y.) at 40°C in absolute darkness. The slides were dried and placed in light-tight boxes in a refrigerator for 4 wk and then developed with Kodak D19 developer. The autoradiographs were stained with hematoxylin and eosin, and afterward the gelatin was destained with dilute McIlvane's buffer. Cartilage samples from each experiment in which cartilage was divided into zonal layers were fixed in formaldehyde, embedded in paraffin, sectioned, and stained as above to assess the histological zonal content of the incubated slices.

Evidence of tissue viability. Viability of the cartilage incubates was demonstrated in rabbit and human samples by their capacity to take up [³H]thymidine. The labeled thymidine and cytidine were used as indicators of cell replication and RNA metabolism, respectively, and showed slightly greater uptake by osteoarthritic than normal control cartilage (Table I). Uptake of ³H-labeled compounds by the rabbit synovial tissue was less than that obtained with articular or ear cartilage. This surprising finding was considered largely the result of a low DNA content. (Table II) and histological evidence by light microscopic examination of inclusion in the samples of considerable connective tissue along with synovial lining membrane.

[2] 1 unit of alkaline phosphatase = 1 mol substrate hydrolysed/min/ml of starting sample.

TABLE I

Uptake of Labeled Thymidine and Cytidine by Incubated Cartilages

	*n**	[*methyl*-^{3}H] Thymidine	[5-^{3}H] Cytidine	[*methyl*-^{3}H] Thymidine autoradiograph
		cpm/mg		*range of counts over cells*‡
1–6-mo-old rabbits				
Articular cartilage	6	1,510±234§	18,930±1,190	4+
Ear cartilage	6	1,085±160	17,230±1,315	3+
Synovia	6	640±119	10,824±980	3+
Patients				
"Normal"	1	750	1,275	—
Fracture	7	820±310	1,350±498	—
Osteoarthritis	13	916±115	2,102±290	—
Rheumatoid arthritis	1	875	1,400	—
Avascular necrosis	2	910±85	1,510±452	—

* Duplicate samples from this number of animals or patients.

‡ See Methods for grade scale.

§ $\bar{x}\pm$SD per unit dry weight of incubated cartilage.

This excess connective tissue increased the sample weight, thus lowering the yield of radioactivity per milligram of tissue. If results are expressed as counts per minute per microgram of DNA content of tissue the values are similar. Under comparable experimental conditions, uptake of both labeled compounds by human osteoarthritic cartilages provided data for specific activities quantitatively similar to that Mankin *et al.* (Table I) (18), with significantly greater uptake of thymidine by osteoarthritic than normal cartilages.

Distribution of silver grain on the autoradiographs of rabbit cartilage was registered 4+ if at least three fourths of the total grains overlaid the cells or lacunae, 3+ if about half the grains were over these sites, and otherwise 1–2+. (Grains were counted in 10 high-power fields of a section from each sample.) Most of the counts were over cells or lacunae for all samples studied (Table I). In adult normal rabbit articular cartilage, however, there was very little uptake of [^{3}H]thymidine, either by chemical or autoradiographic testing, due to the failure of these cells to undergo division in the short-term organ cultures.

RESULTS

Animal data. A consistently positive finding was the output of PPi into the incubation medium by the various

TABLE II

PPi and Alkaline Phosphatase Extrusion by Incubates of Rabbit Cartilage

Tissue	Animal age	Net cumulative PPi extrusion: Incubation time						Tissue content	
		0 h	1.5 h	2 h	3 h	4 h	6 h	Alkaline phosphatase activity	DNA
	mo	*pmol/mg dry wt*						*U/h/mg**	*μg/g**
Articular cartilage	1–6	1‡	21 ±7	56 ±4	88 ±9	90 ±7	67 ±9	0.21±0.16	4.42±1.25
Synovia	1–6	1	1	0	1	2	—	0	2.38±1.21
Ear cartilage	1–6	1	2	0	2	1	0	0	3.9±1.38
Articular, adult whole	24	1	0	1	1	1	0	0.056±0.04	4.3±1.22
Articular, upper zones	1	1	1	0	0	0	0	0.07±0.02	1.1±0.3
Articular growth—prolif‡	1	1	—	—	—	96	—	0.5±0.25	2.5±0.8
HC-cell						±7			
Growth cartilage—resting	1	1	—	—	—	1	—	1.1±0.5	3.1±0.86
prolif		1	—	—	—	252	—	5.3±0.2	4.1±0.7
HC-cell						±5			

Resting, zone of resting cells; Prolif, zone of proliferating cells; HC, hypertrophic cell zone.

* $\bar{x}\pm$SD for incubated cartilage.

‡ Values less than 5 are too low for statistical evaluation in Tables I–III; $n = 9$ for each experimental group.

articular cartilage fragments (Table II), with a peak output reached in 4 h. Controls (ear cartilage and synovial tissue) revealed no detectable output or only trace output of PPi.

Because of the decline of net cumulative PPi output between 4 and 6 h (Table II), enzymic hydrolysis was suspected, and therefore phosphatase activity (pH 10.2) was measured in further experiments on samples of the same fluid in which the PPi was measured concurrently (Table II, Fig. 1). It can be seen that there was a surprising steady output into the medium of phosphatase activity as a function of time by articular cartilage (Fig. 1), whereas none was elaborated by synovial tissue or ear cartilage samples (Table II). Autolysis of cells or leaks from cell membranes as a cause of this phenomenon was interdicted by failure of lactic dehydrogenase to rise in the medium (Fig. 1). Although precise zonal localization of PPi output was impossible due to crudity of histological separation inherent in this preparation, as indicated by Sokoloff[3] (Table II), proliferating cell zones of the growth plate, as well as the growth cartilage portion of the rabbit articular cartilage, were the major cellular constituents in slices that elaborated PPi into the incubating medium. Phosphatase and PPi extrusion showed a positive correlation (Table II). Much higher outputs of PPi and phosphatase by the growth plate than growth portion of articular cartilage were considered at least partly the result of difficulty in selectively sampling the latter site, free of less cellular upper articular zones (Table II). Adult rabbit knee cartilage did not elaborate detectable PPi (Table II).

To ascertain whether enzymic hydrolysis of PPi could be prevented (Table III), several attempts were made

[3] L. Sokoloff. Personal communication.

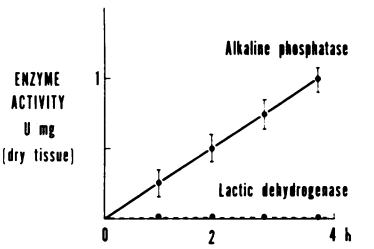

FIGURE 1 Cumulative net extrusion of alkaline phosphatase during incubation of whole young rabbit articular cartilages. No lactic dehydrogenase output was detectable.

with different inhibitors to prevent alkaline phosphatase from being released into the medium. Neither dithiothreitol nor cysteine, potent inhibitors of alkaline phosphatase (Table II), was effective in this dissociation of events; in all instances, output of PPi was inhibited concurrent with the output of alkaline phosphatase. EHDP, 750 μg/ml (3mM), inhibited alkaline phosphatase but not at levels of 150 μg/ml, encountered in human or animal pharmaceutical studies. Actinomycin D at 6.70 μg/ml and 40 μg/ml, as well as colchicine at 0.4 μg/ml and 100 μg/ml, failed to inhibit either PPi or alkaline phosphatase output over a 6-h period (data not shown). Freezing and thawing, as well as closely applied VisKing dialysis membrane (Ethyl Corporation, New York) encompassing the cartilage samples during incubation (Table III), were effective in preventing release of PPi into the cartilage fluid medium. Because PPi was found in the "inside" medium sample after dialysis, it was concluded that the PPi was bound to a nondialyzable complex. Binding was of low avidity inasmuch as after ultracentrifugation of the inside media at 130,000 *g* for 4 h, most of the PPi was found in the supernatant frac-

TABLE III

Effect of Certain Physical Manipulations and Chemical Inhibitors on PPi and Alkaline Phosphatase Elaboration by Rabbit Articular Cartilage

Tissue preparation*	Total PPi elaboration at 6 h	Alkaline phosphatase	[*methyl*-^{3}H] Thymidine
	pmol/mg‡	*U/h/mg*‡	*cpm/mg*‡
Control	62±8	0.18±0.07	1,510±234
Freezing and thawing three times	0	—	
Dialysis (inside/outside)	61±8	0.20±0.06	1,313±370
Supernate from ultracentrifugation 130,000 *g* 4 h	62±9	—	
Dithiothreitol, 10 mM	0	0.035±0.03	750±122
Cysteine, 1 mM	0	0.079±0.01	1,416±150
EHDP, 3 mM	§	0.10±0.03	1,250±210
EHDP, 0.6 mM	§	0.21±0.02	1,394±234

* n = four animals in each experiment.
‡ $\bar{x}$±SD per unit dry weight of incubated cartilage.
§ PPi was not measurable in presence of EHDP.

tion but not the sediment. These findings leave in doubt the state of PPi binding.

Osteoarthritic cartilage samples from 16 patients all put out PPi at about 10 pmol/mg/h (dry wt) or 2.5 pmol/mg DNA/h. In contrast, control patients with the diagnosis of hip fracture, rheumatoid arthritis, or avascular necrosis of the femoral heads have failed to produce any measurable PPi output (Table IV). Phosphatase (pH 10.2) extrusion was significantly elevated above control ($P < 0.01$), although variations were extremely wide. Controls were of comparable ages (Table IV), and cartilage cellularity indicated from DNA content was quite comparable in all control groups.

DISCUSSION

To the authors' knowledge, these data (Table IV) provide the first direct evidence in support of the hypothesis (12) that metabolism of pyrophosphate in local articular cartilages of osteoarthritis is disturbed.

In both the human and rabbit studies, a consistent finding in the laboratory data was simultaneous release of PPi and phosphatase into the incubating media; attempts to dissociate this enzyme release from that of PPi were unsuccessful in rabbits, and so far human cartilages have released minimal phosphatase unless PPi was also released.

A plausible interpretation of the rabbit cartilage data (Table II) is that the release of PPi and phosphatase into the incubating medium is related to processes of calcification. If only cell division and matrix synthesis were involved, the upper slices of the articular samples as well as control synovia and ear cartilage, which also incorporated the [^{3}H]thymidine and [^{3}H]cytidine, should have put out PPi. The results of inhibition experiments bear upon this problem. Thus, although the effect of suppressing phosphatase activity with dithiothreitol (Table III) may have been caused by toxicity to chondrocytes, as shown by the concurrently reduced [^{3}H]thymidine uptake, inhibition of alkaline phosphatase with cysteine or EHDP was completely dissociated from any depression of thymidine uptake by incubated cartilages (Table III). Also, although failure of effect of actinomycin D or colchicine may have been due partly to the shortness of incubation times possible in our in vitro system, relative to time required for maximum action of these inhibitors, the negative data favor the view that the release of phosphatase or PPi does not directly result from altered DNA metabolism or from cell division.

In human cartilage samples, the extension of these interpretations of our data on rabbit cartilages is that osteoarthritic cartilages, but not the other cartilages so far studied, are preparing for remodeling of underlying subchondral bone and cells programmed in preparation to calcify and release phosphatase and PPi. In a previous report, septal mineral deposition was associated with relatively high alkaline phosphatase content of growth cartilage, analyzed by microchemical methods in isolated proliferating and hypertrophic cell zones (28). There is a sharp rise of capillary invasion and initiation of calcification at weight-bearing sites prone to osteoarthritis after age 60 yr in man (29). Also, a high alkaline phosphatase content measured in cartilage at human osteoarthritic sites (30) might be interpreted to indicate a preliminary stage to calcification at sites of subchondral bone remodeling and extension of the tidemark.

There is still the possibility that the PPi released by osteoarthritic cartilages and/or rabbit cartilage has no

TABLE IV

PPi Elaboration by Incubates of Human Osteoarthritic Cartilage

Disease	Patients	Average age	Men	PPi	Phosphatase (pH 10.2)	DNA	Histological grade*
	no	*yr*	*no*	*concn*§	*U*	*mg/g dry wt*	*range*
Synovial fluid							
Normal‡	3	67.5	3	3.6±1.4§		—	—
Osteoarthritis‡	11	61.4	9	12.6±7.0		—	—
Tissue							
"Normal"	2	65.5	1	<1§	0.2±0.1‖	4.1	0–1
Fracture	8	72.1	3	<1	0.1±0.3	4.2±1.1	3–8
Osteoarthritis	16	70.2	12	9.4±2.6	1.78±2.3	3.9±1.3	5–12
Rheumatoid arthritis	3	67.0	1	<1	0.20±0.1	4.5±0.8	—
Avascular necrosis	3	70.0	0	<1	0.33±0.2	4.2±0.4	—

* Mankin criteria (18).

‡ Controls from previous report (12).

§ Concentration in micromolar in synovial fluid and picomoles per hour per milligram dry tissue in incubates.

‖ 10^{-2} U/h/mg dry tissue.

relationship to calcification per se, but represents only intracellular intrinsic metabolic peculiarities related to accelerated matrix repair. Also, implicit in the findings of Pachas (31) and Utsinger et al. (32) is an alternative view that human synovial cells might constitute a source of PPi in synovial fluid. Denuded bone mineral particles pulverized by joint bone-on-bone contact from severe osteoarthritic ulcerations might be an additional source of PPi in synovial fluid. Silcox and McCarty found the highest synovial fluid PPi concentrations in severe osteoarthritis (16), where in our view more extensive ulceration and bone-on-bone contact would be anticipated. Some nonultrafilterable PPi was found by McCarty et al. in human synovial fluid (11). Perhaps this observation is relevant to the fact that rabbit cartilage PPi fails to dialyze (Table III) but yet it separates on ultracentrifugation. No attempt at this point can be made to explain the presence of a bound PPi in the rabbit growth plate cartilage, except perhaps an involvement of a mechanism to calcify.

Finally, the data of Camerlain et al. (33) indicate from clearance rates of ^{32}PPi that the pool size of PPi in knees of a group of patients with osteoarthritis was 0.45 μmol$\pm$0.26 and that turnover was 76% of total PPi/h. Based on the assumption of 6 g of articular cartilage (wet) per knee elaborating (with negligible hydrolysis of PPi) about 1.0 pmol/mg of cartilage (Table III), the predicted output into synovial fluid would be in the range of 1 μmol/h. With further knowledge of kinetics of PPi across synovial membranes, it should be possible to assess whether the currently found extrusion rates of PPi are sufficient to account for the synovial to plasma PPi gradient.

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