Two Latent Metalloproteases of Human Articular Cartilage That Digest Proteoglycan

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Human articular cartilage contains very low levels of metalloprotease activity; the activity in 1 g of cartilage is approximately equivalent to the activity of 1 μg of trypsin. Development of a sensitive assay, based on the digestion of radioactive proteoglycan, has made it possible to study protease activity in 1–2 g specimens of cartilage. Cartilage was extracted with Tris buffer in the cold and with Tris buffer containing 10 mM CaCl₂ at 30 °C. The extracts were passed through Sepharose 6B; two major and two minor metalloprotease activities were detected. A neutral metalloprotease activity, pH optimum 7.4, was found as a latent form of M₉ = 56,000. It could be activated with amionophenylmercuric acetate or trypsin with a resultant decrease of M₉, to 40,000. An acid metalloprotease, pH optimum 5.3, also occurred as a latent form of M₉ = 50,000. Activation converted this to M₉ = 35,000. Removal of calcium ions by dialysis reduced the activity of the neutral enzyme by 80–85% and of the acid enzyme by 100%. Both activities were restored by 10 mM Ca²⁺. Both enzymes were completely inhibited by 1 mM o-phenanthroline in the presence of excess calcium. This inhibition was overcome by 1 mM Zn²⁺ and, to a lesser extent, by Co²⁺. These proteases may be important in the metabolism of the cartilage matrix and in its destruction in osteoarthritis.

In 1976 (1) we discovered that human articular cartilage contains metalloprotease activity capable of cleaving the core protein of cartilage proteoglycan at physiological pH. This activity was blocked by chelating agents and could be restored by Zn²⁺ or Co²⁺. Studies of the enzyme were impeded by the very low levels of activity found. However, it was possible to achieve partial purification and characterization (2) by starting with large quantities of tissue (1200 g from approximately 500 pairs of patellae). Although the proteolytic activities are low, they appear to be important in regulating the slow metabolic turnover of the matrix proteoglycans. Protease activity may also play a central role in degenerative changes of the cartilage, particularly in aging and osteoarthritis. In this connection, it would be very useful to be able to measure enzyme activities in small samples of cartilage (1–2 g). This would permit the study of tissue from a single patient to determine the effect of factors such as age, disease, and drug treatment. Due to the possible presence of tissue inhibitors of proteases in cartilage, it would be necessary to extract the enzymes and to separate them from such inhibitors to be certain that their full activity was expressed.

In this report we describe the extraction and characterization of metalloprotease activities from single specimens of human cartilage which have wet weights of 1–2 g. The sensitivity of existing assay methods has been increased about 30-fold, and methods have been developed for homogenizing and extracting these small amounts of tissue. It was discovered that both neutral and acid metalloproteases are present in cartilage in latent forms. These forms can be activated with mercurials or trypsin, with a concomitant decrease in molecular weight. The metal requirements of these proteases have been established. Although more than 20 metalloproteases of mammalian origin have been reported in the literature, the cartilage metalloprotease is the only one that has an acid pH optimum.

MATERIALS AND METHODS

Enzyme Assay—We have previously described an assay for proteases based on the digestion of proteoglycan monomer prepared from bovine nasal cartilage (3). The monomer is entrapped in polyacrylamide gel beads; the pore size is adjusted to retain monomers, but to permit the escape of digestion products smaller than approximately 200,000 daltons. This assay is used in order to identify proteases in cartilage that can attack the protein core of proteoglycans in the extracellular matrix. The present assay method follows the published description quite closely, except that the incubation time is prolonged and the substrate is labeled with tritium. Labeling was by the method of Montelaro and Rueckert (4). Lyophilized proteoglycan monomer was allowed to swell overnight in 0.3 M sodium phosphate buffer, pH 7.2, at 4 °C. This suspension was then diluted 2-fold to a final concentration of 10 mg of proteoglycan/ml. To 750 mg of proteoglycan in 75 μl buffer were added 10 μCi of [³H]acetic anhydride (New England Nuclear; 50 μCi/mmol). The mixture was stirred for 30 min at 24 °C. The product was dialyzed exhaustively against water at 4 °C, then frozen and lyophilized. Polyacrylamide beads were prepared using this radioactive substrate (3). For enzyme assays, 4 mg of dry beads (approximately 1 mg of proteoglycan, 20,000 cpm) were weighed and placed in a tube. Enzyme and buffer were added to a total volume of 0.2 ml, and incubation was for 18 h at 37 °C. The assay buffer contained 0.06 M Tris/HC1, pH 7.5, 0.01 M CaCl₂, 0.05% Brij-35, 0.02% sodium azide, and 0.2 M NaCl. After incubation, 0.5 ml of water was added, the beads were removed (3), and 0.2 ml of supernatant was transferred to a 20-ml glass scintillation vial. Ten ml of Aquasol (New England Nuclear) were added and shaken. Counting was carried out in a Triacarb Model 3003 (Packard) liquid scintillation counter at 18 °C. Results are expressed as counts/min released per mg of dry bead. No correction was made for the fact that the aliquot was only 2/7 of the total digestion volume. The assay method was shown to give a linear response with time for 18 h and with enzyme concentration, using cartilage enzymes and trypsin (0.3–3.0 ng). At this level of sensitivity there is appreciable noise; reproducibility is approximately ± 8%.

Extraction of Enzymes from Tissue—Human patellae were removed at autopsy and frozen in small plastic bags at −20 °C. Several specimens were also obtained at surgery under sterile conditions, placed
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RESULTS

Crude Extracts.—Due to its elastic nature, cartilage is a very difficult tissue to homogenize. Originally, the VirTis homogenizer was used (1), but this did not thoroughly disperse all of the fragments of cartilage. Sectioning of the cartilage into 20-μm slices with a freezing microtome followed by homogenization in Potter-Elvehjem glass homogenizer was satisfactory but time-consuming. A Polytron homogenizer has proven to give the best dispersion of the tissue in a short time. Brij-35 is added after homogenization to minimize losses of the cartilage proteases.

Bacterial contamination was found to be a serious problem in the early stages of this work. Microorganisms may be introduced at autopsy or during enzyme preparation. *Pseudomonas* and *Corynebacterium* species were particularly prevalent. It was possible to detect microbial metalloprotease production during the course of an 8-h working day, even though the preparations were kept cold and contained azide. These problems were overcome by the introduction of the sterile techniques outlined under “Materials and Methods.” Occasional contaminated preparations were discarded. Several sterile specimens of cartilage were obtained from surgical operations to verify that the activities reported here were not microbial in origin.

Enzyme assays were performed for both neutral and acid metalloprotease activity at the crude extract stage. About 95% of the activity is latent, so the assay included 1 mM aminophenylmercuric acetate to reveal the full activity. Blanks contained o-phenanthroline to assure that only metalloprotease activity was measured. Cathespain D was present in the extracts, but was blocked by azide. The mercurial activator blocks any cysteine proteases that might be present. There are two complications in assaying the crude extracts. The extracts contain proteoglycan, which may compete with the proteoglycan substrate in the beads, and cartilage inhibitors of metalloproteases are present. Attempts to remove the proteoglycan by precipitation or ultrafiltration were not successful. Amicon XM-100 or XM-50 membranes retained the proteoglycan, but the protease activities could not be recovered on either side of the membranes. Cetylpyridinium chloride precipitated the proteoglycan, but the precipitates entrapped the protease activities. It was possible to remove the proteoglycan by chromatography on Sepharose 6B. Fortuitously, this also separated a metalloprotease inhibitor from the enzymes. The full recovery of neutral activity from this column indicated that there is minimal interference in assays of crude extracts by either proteoglycan or inhibitor. However, the acid activity was recovered in increased amount from the column, indicating less reliability of the assay in crude extracts.

A study of extraction methods was conducted using the assay of neutral metalloprotease activity as a guide. Homogenization in Tris buffer yielded both acid and neutral activity. Various second extractions steps were tried: overnight in 1 M NaCl, 2 M MgCl₂, incubation at 37°C, etc. The most useful method was a heat extraction at 60°C in Tris buffer with 10 mM CaCl₂. This method was previously developed to extract collagenase from uterine tissue (6); however, it worked well for cartilage after the calcium ion concentration was reduced from 100 to 10 mM. This second extract recovers an amount of enzyme at least as great as that obtained in the first extract (ratio in 13 experiments was 45:55, Tris extract:60°C extract).

Demonstration of Multiple Enzyme Activities—The two sequential extracts (Tris and 60°C) are made to 1 M NaCl and concentrated on Amicon YM-5 membranes. Variable losses are experienced in this step, probably due to adsorption
on the membranes or other surfaces. The concentrates are then chromatographed on Sepharose 6B (Fig. 1). The Tris extract (Fig. 1A) gives a high peak of protein (not shown) and proteoglycan near the void volume. It is important that the sieve column produce a large separation between the enzyme and the proteoglycan. These compounds tend to interact, spreading the enzyme peaks toward the higher molecular weights. In this event, a second chromatography of the intermediate region is required to produce a clean separation of enzyme and proteoglycan. The major peak of neutral metalloprotease activity (I) emerges slightly beyond the position of the bovine serum albumin marker. Some spreading to the left is due to interaction with proteoglycan. The activity is almost entirely latent (95%) but is activatable by aminophenylmercuric acetate. A smaller peak (II) is found at an apparent $M_r$ = 9,000. This metalloprotease is fully active. However, it is not derived directly from peak I. If the enzyme in peak I is activated and rechromatographed, it emerges at fraction 31 ($M_r$ = 40,000). A further peak (III) containing latent metalloprotease activity emerges in fraction 47, well beyond the column volume. Peaks II and III were generally present in quite small amount (most favorable cases shown) and could not be studied in detail. Serine protease activity was also found in small amount in the region of peaks I and III. The peak of acid metalloprotease activity coincides almost exactly with the neutral activity of peak I and is not illustrated.

Again, the acid activity was almost entirely latent and could be activated with aminophenylmercuric acetate. Both acid and neutral activity could also be activated by treatment with trypsin, 10 $\mu$g/ml for 3 min at 37°C.

Chromatography of the 60°C extracts gives a somewhat different pattern (Fig. 1b). The proteoglycan emerges as a sharp peak at $V_o$, indicating that only intact monomers are present, whereas the Tris extracts contain additional degradation products or small forms of proteoglycan. The major peak I of neutral metalloprotease falls in exactly the position found with the Tris extract. A small shoulder to the right is due to the active form of the enzyme, whereas the main peak is latent enzyme. Peaks II and III are virtually absent from the profile. Again, the acid activity coincides almost exactly with the neutral peak I and is not illustrated.

Properties of the Metalloproteases—Since peak I fell in the same position for both Tris and 60°C extracts, it appeared likely that the same enzyme was being extracted by both procedures. However, the presence of both acid and neutral activities in the same peak suggested that a single enzyme might account for both. A study of pH curves helped to resolve this question. First, it was necessary to activate the latent enzymes so that the measured pH effects would depend only on digestion of the substrate and not on the activation process. The activated enzymes were incubated with substrate and with pepstatin to block aspartic proteases. The activator aminophenylmercuric acetate blocked cysteine protease. The results are shown in Fig. 2. A bimodal curve was obtained with greatly different ratios of acid and neutral activity in the two types of extracts. Furthermore, the activated preparations lost acid activity upon standing for several days. The neutral enzyme then gave a pH curve that fell monotonically from a maximum at pH 7.4 to zero at pH 5.5-6.0. Careful study of seven Sepharose chromatographies showed that, on the average, the acid activity emerged about 1/2 fraction ahead of the neutral activity. Further confirmation that there were indeed two separate enzymes was obtained by additional study of molecular weights.
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The molecular weight of the neutral enzyme was examined both on Sepharose 6B and on Ultrogel AcA-54 columns (Fig. 3). Twenty-eight runs on Sepharose 6B gave an average elution volume of 127.4 ml, which corresponds to $M_0 = 56,900$ in relation to markers of bovine serum albumin, ovalbumin, and cytochrome c. On AcA-54, five runs each of latent and active enzyme gave $M_0 = 56,000$ and 40,000, respectively. Fig. 3 illustrates a separation of mixed active and latent forms; in the other runs, the two forms were studied separately.

The acid enzyme proved more difficult to evaluate. It emerged just slightly ahead (about 0.6 fraction) of the neutral enzyme on Sepharose 6B, suggesting $M_0 = 65,000$. Rechromatography of this peak on Sepharose 6B gave a shift to $M_0 = 50,000$. Other sieve columns were tried, but difficulty was encountered in that the latent acid enzyme almost invariably became self-active upon rechromatography at neutral pH. The use of Sephacryl S-200 at pH 5.3 gave good recovery of the latent enzyme, but some loss of the active form. In this system (Fig. 4), $M_0 = 50,000$ for latent acid metalloprotease and 35,000 for active enzyme.

Metal Requirements—The active forms of both neutral and acid enzymes were produced by treating peak I with 1 mM APMA for 4 h at 37 °C. These preparations were then dialyzed exhaustively against calcium-free buffer. Calcium was then restored at various concentrations (Fig. 5). The activity of the neutral enzyme could never be fully blocked by calcium removal. There was always 15–20% residual activity (five preparations). On the other hand, the activity of the acid enzyme completely disappeared in the absence of calcium. The activity of both enzymes could be completely restored by 10 mM CaCl$_2$, but the neutral enzyme responds to much lower levels of calcium than does the acid enzyme. It should be kept in mind that the assays involve 16–18-h incubations at 37 °C. If the role of calcium is in stabilizing the enzyme, then this long incubation may magnify calcium effects. The diminished enzyme activity at calcium concentrations above 10 mM is due to an effect of ionic strength on the behavior of the bead substrate, not to an inhibition of the enzymes. Zinc did not enhance the small activity of the calcium-free neutral enzyme; rather, 1 mM Zn$^{2+}$ depressed it a further 60%

Evidence that both metalloproteases are zinc enzymes is presented in Table I. In the presence of 10 mM CaCl$_2$, 1 mM o-phenanthroline totally inhibits both enzymes. This indicates that a second metal ion is required in addition to calcium. Zinc restored the activity of both enzymes. Cobalt was less effective; it did not completely restore the activity of either enzyme. In contradistinction to many zinc metalloproteases, the cartilage enzymes are not inhibited by added zinc at a concentration of 1 mM.

Other Properties—That the cartilage enzymes are proteases and not polysaccharidases was shown in two ways. First, the digestion products produced by the peak I enzymes were diazylized in Spectrapor membrane tubing permeable to molecules of mass less than 3500 daltons. Less than 10% of the activity is defined as the amount of enzyme that releases 1 μg of substrate in 1 min. One g of wet cartilage contained 6.8 units of proteoglycan- and 6.0 units of Azocoll-digesting ac-

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**Fig. 4.** Chromatography of acid protease activity on Sephacryl S-200. Pooled fractions from peak I were chromatographed separately as latent or activated forms. Three ml of sample were applied to a column (1.6 × 85 cm) of Sephacryl S-200 (Pharmacia) equilibrated in 0.05 M Tris maleate buffer, pH 5.3, containing 0.2 M NaCl, 10 mM CaCl$_2$, and 0.02% sodium azide. Fractions of 3 ml were collected. Assays were done at pH 5.3 with 5 μg/ml of pepstatin to block cathepsin D and APMA to activate latent enzyme. Markers are as in Fig. 1, with the addition of chymotrypsinogen (25,000).

**Fig. 5.** Effect of calcium on metalloprotease activity. Fractions from peak I were activated with APMA, then dialyzed exhaustively against several changes of calcium-free buffer at pH 7.5. Various levels of calcium chloride were then added to the usual assay system. ●, assay at pH 7.4; ○, assay at pH 5.3.
was dialyzed to remove APMA. The neutral activity was about three times that of the acid activity. The activities in the absence of further additions were set equal to 100. Every incubation contained 10 mM CaCl₂.

| Additions       | Relative activity | pH 7.4 | pH 5.3 |
|-----------------|-------------------|--------|--------|
| None            | 100               | 100    |        |
| 1 mM o-phenanthline | 0                 | 0      |        |
| + 0.1 mM Zn⁺²   | 21                | 25     |        |
| + 0.2 mM Zn⁺²   | 78                |        |        |
| + 1.0 mM Zn⁺²   | 103               | 85     |        |
| + 0.2 mM Co⁺³   | 8                 |        |        |
| + 1.0 mM Co⁺³   | 77                | 66     |        |
| 1 mM Zn⁺²       | 106               | 96     |        |
| 1 mM Co⁺³       | 86                |        |        |

Activity at acid pH. At neutral pH, the values were 4.4 and 11.2, respectively.

From these units and the units obtained with known amounts of trypsin and thermolysin acting on proteoglycan beads, it is possible to estimate the tissue content of the two metalloproteases. One g of wet cartilage contains both neutral and acid activity equivalent to 1 µg of trypsin or 0.1 µg of thermolysin. The acid and neutral activities are found in varying proportions; this value is only an approximation.

Minor Protease Activities—Serine protease activity, inhibitable by phenyl methanesulfonyl fluoride, was detected under peaks I and III of the Tris extracts. Since this activity was low (5-10% of the total neutral activity), no further characterization was attempted. Peak II contained metalloprotease activity that was in a fully active form. It is not derived by direct activation of the neutral enzyme of peak I, as shown by its molecular weight properties (discussed above). It has an apparent $M_r = 9000$ on Sepharose 6B (average of eight runs) and 4000 as measured on ACA-54 and Bio-Gel P-60 (in the same buffer, but with NaCl reduced from 1 to 0.15 M). It is unlikely that these are true molecular weights; interaction of the enzyme with the gels is probable. This form was detected in 8 of 16 Tris extracts and none of seven 60 °C extracts. A single preparation was shown to retain complete activity after removal of calcium by dialysis and to be inhibited by o-phenanthroline with 40% restoration by zinc. Peak III emerged after the column volume of Sephrose 6B, so its molecular weight could not be estimated. It could only be detected after activation by APMA. The cartilage also contains collagenase, another neutral metalloprotease, but this has been reported on elsewhere (8).

**DISCUSSION**

**Advances over Previous Work**—In previous work (1, 2), the smallest batch of cartilage that could be studied was on the order of 100 g, wet weight. With the present methodology, we can now examine as little as 1 g. This improvement is largely due to the increased sensitivity of the assay. In the published method of Nagase and Woessner (3), measurements could be made in the range of 10-50 ng of trypsin. The modified method increases the sensitivity to 0.3-3 ng of trypsin. This gain was achieved by the use of radiolabeled proteoglycan and a 9-fold increase in the time of incubation. Using these methods, it was found that human cartilage contains metalloprotease activities which are equivalent to only 1 µg of trypsin/g of wet tissue. These very low enzyme levels explain why it has been difficult to detect enzyme activity in cartilage homogenates and extracts.

The current study has also brought home the need for sterile techniques. Bacteria are easily introduced from the air and can rapidly produce traces of metalloprotease activity, even when all work is done in the cold. This finding has thrown doubt on our earlier preliminary report of a small metalloprotease in bovine nasal cartilage (9). It is likely that enzyme activity, which seemed to pass through dialysis tubing, was formed by trace amounts of bacterial growth in the external dialysis buffer. We have not been able to find significant amounts of metalloprotease activity in bovine nasal cartilage using our present methods. Since most of our specimens of human cartilage were obtained at autopsy, it was important to obtain sterile samples at surgery to make certain that the enzyme activities reported here were present in fresh tissue. This was found to be the case. Moreover, we have recently completed the study of a large series of surgical specimens of control and osteoarthritic cartilages from the human tibial plateau (13). This shows that the neutral protease is present in normal cartilage and is increased in osteoarthritis. Since the enzyme activity is increased in arthritis, the best starting material for enzyme preparation is cartilage that shows some degree of osteoarthritis, such as fibrillation of the surface and small erosions. Autopsy material is suitable since the cartilage is usually of advanced age and osteoarthritic involvement is frequently noted.

It has not yet been possible to make a complete identification of the neutral protease described here with the activity reported earlier (1, 2). In the earlier studies, the need for activation was not recognized, nor the need for calcium ion; indeed, these studies were done using phosphate buffer. Nonetheless, the neutral protease would probably have become active spontaneously after several purification steps, and it would also have displayed at least 15-20% of its activity in the absence of calcium. The earlier and the present preparations are similar in having a pH optimum between 7.2 and 7.5 and having their activities restored by Zn²⁺ and Co²⁺ after treatment with chelators. The major differences seem to be in $M_r$, which is 40,000 for the active enzyme in the present study and was reported as 26,000 earlier (2). It is possible that the earlier report dealt with the enzyme found in peak II in the present study, an active form of metalloprotease that does not require calcium and is probably retarded by interaction with the sieving column.

Acid metalloprotease activity was also noted earlier (1). There was some evidence for two distinct activities at acidic pH, both inhibitable by o-phenanthroline. However, in the present study, only a single peak was detected with an optimum at pH 5.3. Again, the latency and calcium requirement had not been noted earlier. But restoration of activity after chelator treatment with Zn and Co had been noted earlier.

**Source of the Metalloproteases**—It seems quite probable that the metalloprotease activities of the cartilage are produced by the resident chondrocytes. Activity is found not only in diseased cartilage, but also in cartilage from young adults that has no evidence of arthritic changes. Recently, McGuire et al. (11) have shown that human chondrocytes in culture produce a metalloprotease that can digest proteoglycans. Similar metalloproteases that digest proteoglycan at neutral pH and that occur in latent forms activated by mercurials and trypsin have been found in the culture medium of rabbit (12, 13) and bovine (14, 15) chondrocytes. The work of Morales and Kuettner (16) is particularly instructive. They showed that bovine chondrocytes produce metalloprotease under conditions in which the cell phenotype is maintained, e.g. the cells produced...
only type II collagen. However, the bovine enzyme had \( M_r = 33,000 \) and did not undergo a change in weight upon activation. The enzyme produced by immature rabbit cartilage in organ culture (16) is closely similar to the human neutral metalloprotease in having \( M_r = 53,000 \) for the latent form and 40,500 for the active form. Our laboratory is the only one in which metalloprotease has been successfully extracted directly from cartilage tissues.

Relation to Other Metalloproteases—While bacterial metalloproteases have been known for a long time, mammalian metalloproteases capable of endopeptidase action are of relatively recent discovery. Collagenase was first found in 1962 (17) and shown to be blocked by EDTA. Evidence that collagenase is a zinc enzyme appeared in 1973 (18); calcium is required as well. During the 1970s a number of additional metalloproteases have been described. These have most commonly been assayed on gelatin, proteoglycan, Azocoll, and casein. In general, these enzymes are capable of degrading components of the connective tissue matrix, they are secreted by cultured cells, and they commonly occur in latent forms that can be activated by mercurials and trypsin. A second group of metalloproteases is found intracellularly, commonly in association with microsomal membranes. The rabbit kidney metalloprotease (19) is typical of this group. These enzymes require zinc, but not calcium; they are inhibited by 1 mM \( Zn^{2+} \). Latency and secretion of such enzymes have not been reported.

The two major cartilage metalloproteases reported here fall into the first group, capable of attacking matrix macromolecules. Recent work suggests that the latency phenomenon is due to synthesis of the enzymes in a proenzyme form, rather than to enzyme-inhibitor complex formation (20). The neutral activity from cartilage has properties similar to those reported for the rabbit cartilage enzyme (16) and rabbit bone enzyme (21). The properties of all three enzymes are not yet known in sufficient detail to judge just how closely related they are.

Further characterization of the acid metalloprotease is of interest because this type of activity has not hitherto been described in mammalian tissues. There is a well known group of acid metalloproteases found in microorganisms, including \textit{Penicillium roqueforti} and \textit{caseiicum} and \textit{Aspergillus oryzae} and \textit{sojae} (22). These have molecular weights of 20,000 and contain zinc. The cartilage enzyme differs from these enzymes in having a higher molecular weight and occurring in a latent form. Recently, Galloway et al. (23) have described further details of the rabbit bone metalloprotease. The active form of this enzyme has a very broad pH optimum which is almost flat from pH 5.5 to 9.0. However, the cartilage acid metalloprotease is distinguished from this enzyme in having a sharp pH optimum near 5.3.

We believe that a thorough understanding of the cartilage metalloproteases will be important in understanding the physiology of the extracellular matrix and its degradation in disease processes. The method of direct extraction of these enzymes, used in conjunction with tissue culture studies of enzyme production, would be particularly useful in studying the effects of drugs on joint disease.

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J. Biol. Chem. 1984, 259:3633-3638.

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