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The Source of Synovial Fluid Alkaline Phosphatase.* (31548)

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Most of the proteins in synovial fluid have been identified with those of serum(1,2). Because of this, as well as similarities in the distribution of electrolytes and other constituents, synovial fluid is often considered to be an ultrafiltrate of serum(3). The passage of serum proteins into the synovial cavity is, however, a selective process which excludes such proteins as macroglobulins and blood clotting factors(2,4). It is doubtful that all synovial fluid proteins are derived from serum. For example, the observation that the specific activity of alkaline phosphatase in bovine synovial fluid is often 100 times higher than in serum(3) would lead one to conclude that the synovial fluid enzyme is not derived from serum. Histological evidence supports the view that the phosphatase is a product of the surrounding connective tissues(5).

Electrophoresis(6-9), sucrose density gradient ultracentrifugation(10) and protein fractionation techniques(11) were used in an effort to establish a relationship of bovine synovial fluid phosphatase with a comparable serum or connective tissue enzyme. The investigations reported here illustrate the use of agar-gel electrophoresis and sucrose density gradient centrifugation for the separation of alkaline phosphatases. Complications resulting from isolation procedures and the breakdown of enzyme and tissue extracts are discussed because of their importance to the general problem of enzyme technology.

Methods. Samples of synovial fluid, free of contamination with blood, were obtained from the astragalotibial joints of yearling heifers or steers.‡ After clarifying the fluid by centrifugation at 1000 × *g* for 45 minutes, hyaluronidase§ (2 mg per 10 ml of fluid) was added to depolymerize the hyaluronic acid. The depolymerized solution was used for subsequent experiments.

The alkaline phosphatase of synovial fluid was purified by a combination of $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography on DEAE- and TEAE-celluloses‖(11). This scheme could not be successfully applied to serum probably because it is a more complex mixture of proteins and contains phosphatases derived from a number of tissues (12).

Because of the low specific activity of alka-

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§ Nutritional Biochemicals Corp., Cleveland, Ohio (approx. 300 U.S.P. U/mg).

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line phosphatase in serum, a preliminary fractionation was performed on a column of Sephadex G-200.¶ Elution of the column with 1 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0, yielded 3 protein peaks; the phosphatase activity was associated with the second peak. The enzymatically active eluates were concentrated by pressure dialysis in collodion bags** against 0.05 M Tris-HCl buffer, pH 8.0, which was also 10^{-3} M in $MgCl_2$.

Scrapings of bovine articulating cartilage were extracted for 24 hours at 5°C with 10 volumes of 0.05 M Tris-HCl buffer, pH 7.6, per gram of material. The extract was centrifuged and the supernatant was concentrated by pressure dialysis according to the procedure outlined for serum. To insure that only cartilage tissue was present in the scrapings, samples were examined histologically. The tissue was also examined histochemically for phosphatase activity(13,14).

The technique of Martin and Ames(10) was used for the density gradient sedimentation studies and was standardized with crystalline bovine serum albumin. Samples containing 20 mg of protein per ml were layered on the sucrose gradients which were subsequently centrifuged at 37,652 rpm for 16 hours. The tubes were pierced and successive 10-drop fractions collected. Total protein was measured by the method of Warburg and Christian(15) and alkaline phosphatase activity was assayed with p-nitrophenyl phosphate as substrate(11).

Agar-gel electrophoresis was performed according to the method of Wieme(9). The plate was prepared by pipetting 15 ml of hot 1.2% agar,†† dissolved in 0.05 M barbital buffer, pH 8.2, onto a 3¼ × 4 inch glass plate. After solidification, sample wells were formed with a disposable Pasteur pipette and subsequently filled with the appropriate solutions. The electrophoretic separation was conducted at 200 volts for 1 to 1½ hours at 5°C. Visualization of the enzymatic activity was accomplished by the method of Hodson *et al*(16) with α-naphthyl phosphate and p-chlor-o-toluidine diazonium chloride. Non-specific staining of protein by the diazonium salt was determined on a separate sample by adding a phosphatase inhibitor, 0.01 M EDTA, to the incubation medium.

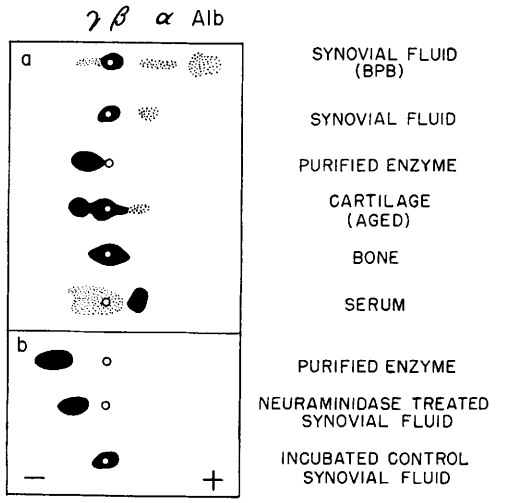

FIG. 1a. Agar-gel electropherogram showing migration of alkaline phosphatases from various sources. Topmost synovial fluid pattern, used as a marker, was stained with bromphenol blue prior to electrophoresis; the remaining samples were stained for phosphatase activity(12). Enzyme activity is represented by solid black regions while non-specific staining is indicated by stippled pattern. No effort is made to give quantitative degrees of enzyme activity.

FIG. 1b. Electropherogram of purified enzyme (11) and neuraminidase treated synovial fluid after development for phosphatase activity(16). The samples were allowed to migrate longer than those in part A in order to resolve the two cathodically migrating forms.

Results and discussion. Preliminary investigations indicated that neither vertical starch-gel electrophoresis(6,7) nor acrylamide disc electrophoresis(8) resolved the phosphatases from the sources considered. Distinct differences in electrophoretic mobility were apparent in agar gel(9). Fig. 1A shows that the alkaline phosphatase activity of unfractionated synovial fluid remained at the origin while the purified enzyme migrated cathodically. The change in mobility resulted after chromatography of the enzyme on TEAE-cellulose(11) and caused some concern about structural alteration of the enzyme. Sedimentation of the purified enzyme in a sucrose gradient showed that the alteration was not significant enough to be detected by this tech-

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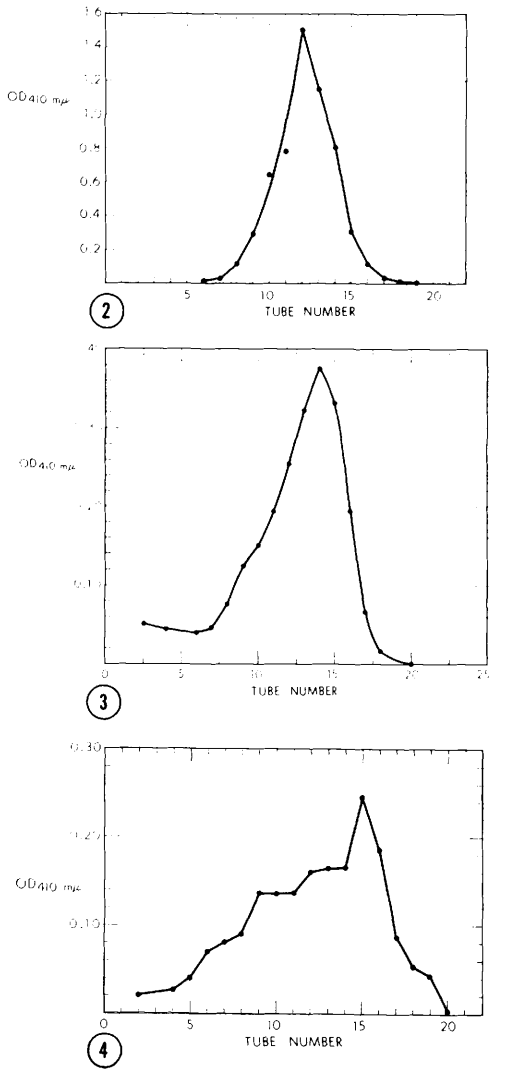

FIG. 2. Ultracentrifugal sedimentation in a sucrose gradient of synovial fluid alkaline phosphatase activity.

FIG. 3. Ultracentrifugal sedimentation in a sucrose gradient of cartilage alkaline phosphatase activity.

FIG. 4. Ultracentrifugal sedimentation in a sucrose gradient of bovine serum alkaline phosphatase activity.

nique(11). A single symmetrical peak of enzyme activity was found with unfractionated synovial fluid after ultracentrifugation in a sucrose gradient, Fig. 2, and only one component was observed after electrophoresis, Fig. 1A. It, therefore, appears that only one molecular form of phosphatase is present in synovial fluid.

The effect of ion-exchange resins on the enzyme is pertinent since it concerns the problem of identification of isozymes in biological materials. Boyer described a new phosphatase isozyme in serum which became discernible only after fractionation of serum by a procedure including chromatography on TEAE-cellulose(17). The current experiments with synovial fluid phosphatase indicate that caution must be exercised in these identifications since treatment with ion-exchange resins may result in modification of proteins and, consequently, electrophoretic artifacts.

A cathodically migrating phosphatase component was observed in aged cartilage extracts while non-migrating enzymes were found in bone and in all fresh extracts of cartilage, Fig. IA. When the fresh extracts were allowed to age at 5°C for a week or longer, the cathodically migrating activity appeared. This change occurred even in the presence of antibiotics as streptomycin, suggesting that bacterial contamination was not involved. The altered mobility, therefore, may be the result of endogenous hydrolase activity(18). Moss, for example, has shown that the heterogeneity of intestinal phosphatases is the result of modification of a single enzyme rather than due to the presence of isozymes (19). Additions of chymotrypsin or trypsin (1 part per 100) to fresh cartilage extracts resulted only in loss of activity without affecting electrophoretic migration. On the other hand, treatment of synovial fluid with neuraminidase in the manner outlined by Moss (20), converted the phosphatase into a cathodically migrating form, Fig. 1B. It appears from these results that the loss of a small acidic peptide or sialic acid could have occurred during purification of the synovial fluid enzyme on the TEAE-cellulose. A similar phenomenon was demonstrated to occur during purification of prothrombin(21).

Serum, which contributes most of the proteins to synovial fluid, contains alkaline phosphatase(s) migrating in the α-globulin region, Fig. 1A. Non-specific reactions of the diazonium compound with protein were found at the origin and cathodically to it, Fig. 1A. The difference in electrophoretic migration of

serum alkaline phosphatase(s) and the synovial fluid enzyme might be explained by the influence of various components in these two fluids, *e.g.*, proteins, lipids or mucopolysaccharides. Mixing synovial fluid with serum, however, yielded zones of activity representing each of the enzymes of these solutions alone. Had the enzymes been affected by their environment, a change of mobility should have been observed. Some serum enzymes have been shown to increase in activity after sonication because of the rupture of lipid-protein complexes(22). Sonication of serum and synovial fluid, however, altered neither the mobility of these phosphatases nor their activity.

Sucrose gradient ultracentrifugation of fresh cartilage extracts shows a slight asymmetry of the activity peak, Fig. 3, indicating heterogeneity of phosphatases. Serum, on the other hand, was resolved into at least 4 peaks, Fig. 4; this resolution is better than that achieved by electrophoresis for this particular enzyme source. The present studies demonstrate that the sedimentation of the major phosphatase component of synovial fluid, cartilage and serum is similar and, therefore, these enzymes may be of the same size.

It appears to us, on the basis of the electrophoretic and sedimentation studies, that the enzyme of synovial fluid is derived from hyaline cartilage of the joint rather than from serum. Despite histological evidence(5) indicating that the synovium is the source of synovial fluid phosphatase, extracts of these tissues were devoid of any activity.

Summary. Agar-gel electrophoresis shows that synovial fluid alkaline phosphatase migrates differently from that of serum but similar to the enzyme of fresh cartilage and bone extracts. It is concluded that the synovial fluid enzyme is derived from the articulating cartilage and not from serum. Purification of the synovial fluid enzyme by a method involving chromatography on TEAE-cellulose leads to modification of the enzyme that is accompanied by a change in electrophoretic mobility but not in its rate of sedimentation in a sucrose gradient. Changes in mobility of phosphatases occurred as the result of aging cartilage extracts or treating synovial fluid with neuraminidase. Chymotrypsin and trypsin, which do not alter the mobility, inactivate the phosphatase of cartilage.

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