Meetings

Collagenase

The first Interdisciplinary Symposium on Collagenase, held at Columbia University, College of Physicians and Surgeons, 5 February 1970, was designed to acquaint clinicians with the nature, mechanism, and rationale underlying the use of the enzyme and to reveal to the basic scientists the practical applications that have been developed.

In an introductory lecture Ines Mandl (Columbia University) pointed out that one-third of all the protein in the mammalian organism is collagen, which is the main constituent of skin, tendon, and cartilage and is the organic component of teeth and bone. Enzymes capable of degrading collagen thus assume importance in remodeling processes, in laboratory investigations involving collagen, and in clinical applications where collagen-rich structures are implicated. Some 30 years ago such an enzyme was first found in the medium of certain clostridia. Later, one species—*Clostridium histolyticum*—was recognized as the best source, and extensive screening produced strains capable of elaborating the enzyme in good yield with minimum toxicity.

No collagenase could be demonstrated in mammalian tissues until recently. During the last few years two important developments have taken place. The bacterial collagenase has emerged from a laboratory curiosity to an easily available drug; it has proved its worth in massive clinical tests and has been suggested for additional uses both clinically and in the laboratory. Mammalian collagenases have been detected by elegant new techniques and appear to play a role in physiological and pathological processes. The time thus seemed appropriate for an evaluation of the current status of collagenolytic enzyme research.

Twenty-seven invited speakers discussed their findings in four consecutive sessions. Only the highlights can be cited here. Most of the papers dealt with *Clostridium histolyticum* collagenase, but Jerome Gross (Harvard University) summarized present knowledge of collagenases of animal origin. At the outset he stressed that changes in the polymerizing properties of fibrils or subunits do not constitute direct evidence for collagenase production because subtle changes in environment or structure which do not affect the main peptide chain may alter fibril formation. Only cleavage of native collagen under environmental conditions in which denaturation does not occur is acceptable proof for collagenase activity. Furthermore, only if the action of the enzyme is greater than that of trypsin, can nonspecific action on peptide appendages be ruled out. Since the stability of collagen in solution below *p*H 5 is lowered, proteolysis at temperatures between 32° and 37°C at *p*H below 5 is likely to be the result of slow denaturation, in which case trypsin at a *p*H above 7 would also lyse the substrate. He further warned that cleavage of synthetic oligopeptides which are used to measure bacterial collagenase titers is not evidence for animal collagenase since other peptidases in animal tissues have this ability.

Although the two activities often occur together, Harper and Gross showed that, in the case of tadpole collagenase, these activities are due to different enzymes, and they separated them by gel filtration and acrylamide electrophoresis. No collagenolytic activity as defined above has been detected in extracts of tissues from tadpoles, from the postpartum uterus, or from resorbing bone although these tissues in continuous culture produce the enzyme. It is thus suggested that collagenase is produced by de novo synthesis during culture and is not stored or present in appreciable amounts in vivo. This was also borne out by the complete elimination of activity in the presence of puromycin. Collagenases have been isolated from the culture medium of living tissues from normal and diseased human skin, from the edges of healing skin wounds, central granulation tissues, the uterus (both postpartum and during human endometriosis), inflamed human gingiva, inflamed synovial tissue from patients with rheumatoid arthritis, regenerating newt limb, and tadpole tail fin and back skin. In all cases freezing and thawing the tissue prevented appearance of activity. The ranges of *p*H (7 to 9) are similar to that of bacterial collagenase, with no activity below *p*H 5; the action of tissue collagenase is, however, much more limited; it cleaves the collagen molecule into two pieces only, usually 25 and 75 percent of the intact molecule. In contrast to the intact collagen, these pieces are soluble in physiological saline at room temperature; once dissolved, the fragments denature spontaneously at body temperature and can then be reduced to small peptides by the collagenase or by nonspecific proteases. Human or bovine serum, which has no effect on bacterial collagenase, blocks activity. It appears that production of animal collagenases is under close cellular control and that the enzymes are not lysosomal but synthesized and released on demand. Synchronization of degradative and biosynthetic mechanisms undoubtedly controls growth, morphogenesis, and connective tissue repair. Evidence for more stable collagenolytic activity remains meager, but recently Eisen and Gross did reveal some activity in insoluble residues of extracted, frozen, smashed powder from tail-fin tissues of tadpoles that had been treated with thyroxin for 6 days; similar activity had been found in bone extracts, and Lazarus *et al.* at NIH have extracted a collagenolytic enzyme from human leukocytes.

In addition to the obvious interest of the role of animal collagenases in physiological and pathological processes, enzymes with such limited yet well-defined specificity are ideal tools for investigation of collagen structure. Karl Piez (NIH) reported that he used tadpole collagenase in experiments designed to answer three important questions about the molecular structure of collagen. Although it has been known for a long time that collagen consists of three chains, it had not been established whether all three chains were of the same length or whether they were folded in some complex way. Cleavage by the enzyme at a single point gave equivalent fragments of 24,000, 72,000, and 145,000 molecular weight. The two smaller pieces could each be

resolved by carboxymethyl-cellulose chromatography into two major components with a ratio of 2 : 1, the same ratio as that of α_1 chain to the α_2 chain in the intact, rat skin collagen substrate. Amino acid analysis showed that one of the 72,000-molecular-weight fragments plus one of the 24,000-molecular-weight fragments equaled the composition of α_1 and the other that of α_2.

Since the molecular weight ratio is 3:1 in all cases, cleavage of all three chains must have occurred in equivalent places. The second question posed was whether all chains ran in the same direction. Amino- and carboxyl-terminal analysis of the fragments showed that all of the small pieces had NH_2-terminal leucyl or isoleucyl residues which are not present in uncleaved collagen; the smaller piece thus represents the COOH-terminal end in all three chains and all run in the same direction. This was confirmed by comparison with cyanogen bromide peptides of known composition from the COOH-terminal end. Finally, in his examination of the tadpole-collagenase fragments he was able to answer the question whether cross-linking sites existed in both parts of the molecule or only at the NH_2-terminal end. The presence of a molecular weight fragment of 145,000 molecular weight and the complete absence of a 48,000-molecular-weight class proved that dimers formed by interchain cross-links in the larger three-quarter NH_2-terminal piece only; there were no dimers of the 27,000-molecular-weight species, therefore no intermolecular cross-links exist near the COOH-terminal end of the α chains of soluble rat skin collagen. Piez also reported the use of bacterial collagenase as an aid in the complete sequencing of the amino acids in the α_1 and α_2 chains. In particular, both P. Bornstein and W. Butler obtained, by further cleavage of cyanogen bromide products representing 120 amino acids at five positions, peptides which were small enough for sequencing by standard methods and which, at the same time, afforded further insight into the specificity of bacterial collagenase. Although cleavage occurred at the expected sites in most cases, the presence of charged residues appeared to inhibit hydrolysis. If there was more than one imino acid in the region, cleavage seemed more likely. Interpretation of observations made at the NIH laboratories as well as some by Stark and Kühn in Germany indicate that collagen contains regions of varying susceptibility to collagenase and that the enzyme works near the ends of chains where they become unraveled rather than in the helical body of the chain. This would also explain why the NH_2-terminal end is not attacked initially. The high content of imino acids in the 50 residues that follow immediately the short cross-link region accounts for a highly stable helical conformation which renders it resistant to collagenase in spite of the susceptibility of some of the sites after denaturation.

Both tissue collagenase and bacterial collagenase were also applied by Carwile LeRoy (Columbia University) in his study of antigenic and immunogenic sites of collagen and a collagen-like protein that contains hydroxyproline in human plasma. Greater knowledge of the antigenicity of collagen may reveal ways to overcome the potential limitation of prosthetic and transplant devices. Michaeli at the Kaiser Foundation Laboratories has indicated that a major antigenic determinant of collagen is located at the NH_2-terminal end of the α_2 chain. LeRoy used a collagenase derived from rheumatoid synovium to cleave calf skin collagen without destruction of the helix. The NH_2-terminal (75 percent) fragment was separated from the COOH-terminal (25 percent) product by ammonium sulfate fractionation monitored by gel electrophoresis. Full antigenicity was retained in the larger NH_2-terminal piece; no antigenic determinants were detectable in the smaller piece. Clearly the older view that antigenicity resides only in the telopeptide appendages is no longer tenable. This was found also by Levi, Manahan, and Mandl who were able to produce antibodies to highly purified collagen free of telopeptide appendages. Bacterial collagenase was used by LeRoy to degrade the hydroxyproline-containing protein from plasma with a view to applying the resulting peptides to immunological studies of human collagens. The dominant peptide, which was separated from the low-molecular-weight digestion products and was purified by ion-exchange chromatography and high-voltage paper electrophoresis, had a molecular weight of approximately 5000 and contained 26 percent glycine, 12 percent hydroxyproline, and 12 percent proline, suggesting similarities in structure between the parent protein and collagen. Sidney Udenfriend (Roche Institute of Molecular Biology) found bacterial collagenase that was free of all nonspecific proteolytic activity an ideal tool for studies of the biosynthesis of collagen. Using chick embryo material, grown under nitrogen to exclude hydroxylation and under oxygen when hydroxyproline is formed, he showed that in both cases collagenase-susceptible peptides of different sizes are produced. The completely formed protocollagen α chain can accumulate and be hydroxylated later, but in addition proline-containing peptides with a molecular weight as low as 10,000 to 20,000 can be recognized by the collagen proline hydroxylase. Most workers have studied the progression of collagen biosynthesis by hydroxyproline excretion, which is the final step; but use of collagenase allows independent study of the primary ribosomal event by the appearance of collagenase-susceptible fragments. A marker, radioactive tryptophan, was incorporated in the system to ensure that only collagen building blocks were attacked and that none of the proline peptide degradation products was derived from other extraneous proteins. During the early phases of fibroblastic tissue culture, collagenase-susceptible material was produced before hydroxyproline was formed. When labeled puromycin was added, biosynthesis was stopped but hydroxylation proceeded. Peptides containing the labeled puromycin and hydroxyproline on the same fragment of approximately 10,000 to 20,000 molecular weight were obtained and cleaved by collagenase; the subsequent release of both the puromycin and hydroxyproline peptides proved that hydroxylation can occur at different intermediate stages of collagen chain formation.

Because of the selective action of collagenase which does not damage cell membranes, this enzyme may be used as a cell dispersion agent, especially in tissue culture. E. Lasfargues (Institute for Medical Research, Camden) reviewed the many uses in the tissue-culture field including his own development of three mammary cell lines now applied to the study of mouse mammary tissue virus and primary cultures of human mammary carcinoma. Organs with large amounts of collagen could be incubated with collagenase for longer times and at higher concentrations than with other proteolytic enzymes without loss of cell viability. Retention of membranes in an undamaged state would allow investigations of membrane-budding viruses and immunoassays in which cell membrane receptors are involved. Lasfargues also reported the ap-

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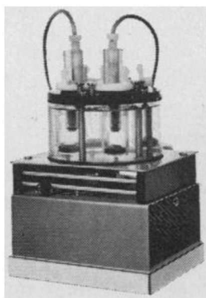

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plication of human skin fragments dispersed by bacterial collagenase as a multiple graft system to speed up the healing of extensive burns. Dispersed skin cells in a freely flowing suspension were spread out evenly over the complete surface of the wound in small circular drops. After a few hours, skin regeneration had started from a number of foci which gradually became confluent. It was pointed out that this procedure might be worthy of further trials. In another experimental use cell dispersion with collagenase enabled Carlton Blackwood (Columbia University) to successively transplant an ovarian papillary serous cystadenocarcinoma of human origin beyond 40 transfer generations in rats and hamsters. Without collagenase, increasing amounts of connective tissue accumulated between tumor nodules; and serial transplants could not be carried out beyond three or four transfer generations. Removal of the collagen rendered the tumor transplantable indefinitely.

Leonard Shulman and his associates (Harvard) treated tooth allografts with bacterial collagenase prior to transplantation. This procedure dissolved the collagen fibers in the periodontal ligament and thus prevented early rejection caused by the immunogenicity of the periodontum without damaging the tooth cement. When a tooth is transplanted within the same mouth it reattaches to alveolar bone within 3 weeks and survives indefinitely; but tooth transplants between individuals do not reattach normally and are ultimately lost because of root resorption. It was demonstrated that, in the absence of prior treatment with enzyme, at 3 weeks there is extensive lymphoid infiltration leading to rejection of the foreign periodontal ligament. Comparison of control allografts and enzyme-treated allografts in rhesus monkeys after 3½ months showed that enzymolysis of the periodontal ligament before transplantation significantly reduced inflammation after transplantation and increased ankylosis leading to prolonged survival of the tooth allograft.

Two other applications of collagenase of great potential use to human patients were reported, though as yet both are restricted to experimental animals. Bernard Sussman (Howard University) used bacterial collagenase to dissolve the protruding cartilage which, through compression of the nerve root, causes severe pain in herniation of the intervertebral disk, the condition commonly referred to as slipped disk. This nonsurgical decompression of the nerve root is possible because of the selective enzymatic dissolution of the disk which assures a margin of safety not shared by common proteolytic enzymes. Sterile collagenase was injected directly into the nucleus pulposus of dogs. The cartilage was dissolved without any damage to the surrounding tissue. All dogs walked immediately after recovery from anesthesia and showed no evidence of dysfunction of any sort. Preliminary trials in vitro against tissues removed from human patients in the operating room or at autopsy showed similar favorable action. Collagenase mediated complete destruction of the nucleus pulposus and major dissolution of the fibrocartilage, the tissues that constitute the bulk of the offending mass in clinical disk herniation while hyaline cartilage is usually spared and osseous effects are insignificant.

Dogs were used by Frank Longo and John Lattimer (Columbia) in their evaluation of collagenase as an adjunct in cryoprostatectomy. With increasing life expectancy, more poor-risk patients unsuitable for conventional surgery present themselves with obstruction of the bladder caused by benign or malignant enlargement of the prostate gland. Cryoprostatectomy—which is fast, requires no or little anesthesia, and results in negligible blood loss or trauma—has many advantages for patients of advanced age. In this otherwise highly successful procedure, the single most frustrating complication has been the retention of slough which plugs up urinary passages and prevents elimination. Direct injection of collagenase into the prostate glands of 15 dogs before the cryoprobe was put in place gave the desired result of removing the slough and retaining normal urinary function without producing demonstrable histologic damage to vital tissues. In addition, several commercially available enzymes were tested for the purpose of degrading or decomposing "cryoslough" from patients after cryoprostatectomy. Collagenase was significantly superior to the other agents tested. In a concentration of 0.1 percent total dissolution was accomplished in 18 hours.

In the clinical sessions, good therapeutic effects were reported in more then 1500 patients who were given topical applications of bacterial collagenase in an ointment base for debridement of second- and third-degree burns prior to skin grafting and for the treatment of dermal ulcers. Ingo Mazurek (Knoll A.G.) summarized results

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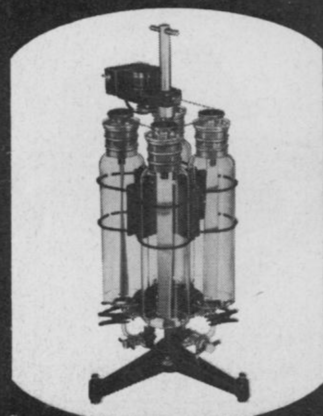

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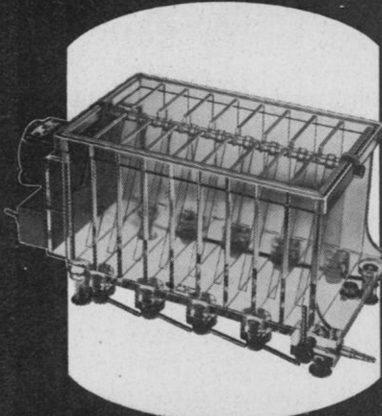

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obtained by 69 investigating physicians in West Germany. Of 1356 patients treated, 78 percent had leg ulcers. In children, burns were the most frequent indication. In 20 percent of the patients, the enzyme took effect within 3 days, in 97 percent within 14 days. Overall, a satisfactory result was obtained in 80 percent of all patients. Walter Zimmermann (University of Freiburg, Germany) reported that, in almost 400 burn cases, not a single keloid was formed and none of the wounds contracted in contrast to the relatively high incidence of both with other treatments. The various presentations conveyed the general impression that, after some 20 years of animal and clinical experimentation, a valuable addition to the armamentarium of topical drugs available for ambulatory and institutionalized patients had emerged.

INES MANDL

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Forthcoming Events

October

20–23. American **Vacuum** Soc., 7th annual, Washington, D.C. (Miss D. M. Hoffman, RCA Labs., David Sarnoff Research Center, Princeton, N.J. 08540)

21–23. Society of **Mining Engineers** of the American Inst. of Mining, St. Louis, Mo. (J. C. Fox, 345 E. 47 St., New York 10017)

21–23. **Planetarium Teachers**, East Lansing, Mich. (V. D. Chamberlain, Michigan State Univ., Talbert and Leota Abrams Planetarium, East Lansing)

21–23. **Ultrasonic** Symp. (G-SU), San Francisco, Calif. (W. J. Spencer, Bell Telephone Labs., Allentown, Pa. 18103)

21–24. National Assoc. of **Biology Teachers**, Denver, Colo. (J. P. Lightner, NABT, 1420 N St., NW, Washington, D.C. 20005)

21–24. Society of **Photographic Scientists and Engineers**, Washington, D.C. (H. J. Hall, 10 Maguire Rd., Lexington, Mass. 02173)

22–23. National Acad. of **Engineering**, Washington, D.C. (National Acad. of Engineering, Editorial Office, 2101 Constitution Ave., NW, Washington, D.C. 20418)

22–24. American Soc. for **Aesthetics**, Boulder, Colo. (J. R. Johnson, ASA, 1150 East Blvd., Cleveland, Ohio 44106)

22–24. **Gerontological** Soc., 23rd annual, Toronto, Ont., Canada. (E. Kaskowitz, GS, 660 S. Euclid, St. Louis, Mo. 63110)

23–24. **Biological Sonar and Diving Mammals**, 7th annual conf., Menlo Park, Calif. (T. C. Poultier, Biological Sonar Lab., Stanford Research Inst., Menlo Park 94025)

25–28. National **Lubricating Grease** Inst., 38th, Atlanta, Ga. (C. V. Pickell,

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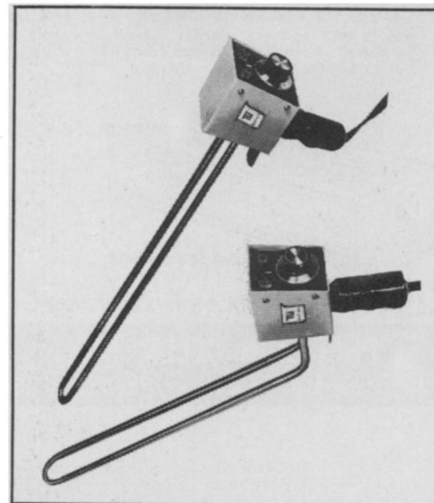

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