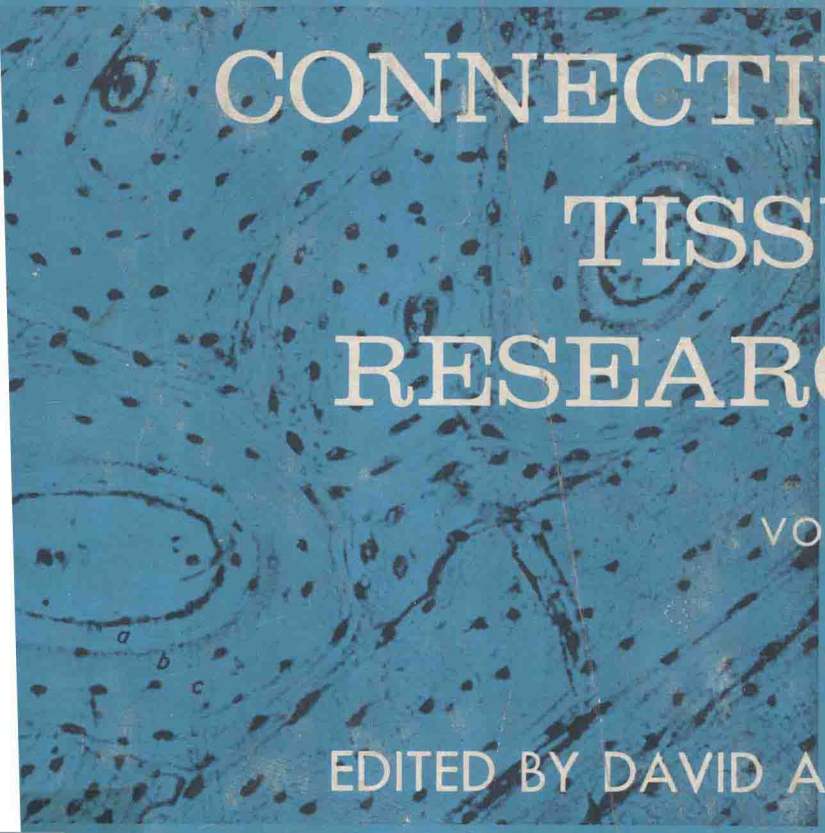
INTERNATIONAL REVIEW OF CONNECTIVE TISSUE RESEARCH



VOLUME 3

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International Review of Connective Tissue Research

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Preface

In the course of editorial endeavors to find material suitable for this serial publication the attention of prospective contributors is drawn to a somewhat nebulous group of suggestions bearing the rather grandiose title of "Editorial Policy." These suggestions are not very demanding of the authors but they do contain a definite mandate to prepare a critical review of a chosen subject rather than a bibliographical survey of the literature. It is one of the more pleasant aspects of an editor's task to find how much more interesting contributors can make their articles by acceding to this request. The contributions to the present volume demonstrate this very clearly over a wide range of topics.

Limitation of the number of contributions to this volume has not only made it possible for the authors to include much of the background material which is essential for an appreciation of their subjects but also to discuss their own contributions in a critical manner. The spread from physical chemistry to oral pathology helps maintain the broad approach which has been a feature of the two previous volumes.

Once again the Editor wishes to record his indebtedness to the contributors for their cooperation in the preparation of this volume and to Dr. J. E. Wilkinson for compiling the subject index.

D. A. Hall

August, 1965

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J. A. PIERCE

Copper and Connective Tissue
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The Histochemistry of the Connective Tissues

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I. Introduction

Connective tissues are composed of cells and intercellular substances. The intercellular substances in general may be categorized into collagenous tissues, elastic tissues, and ground substances. The functions of cells and the types of intercellular substances produced vary with

anatomic site and function of the part. Connective tissues most importantly serve portative, supportive, defensive, storage, and reparative functions; thus, (1) a loose type of connective tissue serving a portative function generally exists between the vascular system and tissue cells; (2) either cartilagenous or ossified tissue occurs in skeletal sites providing a firm supportive function, or predominantly collagenous tissue in tendons, ligaments, or periodontal membranes serves a resilient supportive function for these organs; (3) reticuloendothelial cells throughout the body and particularly in lymph nodes and spleen (a) participate in defense measures by phagocytosis and the production of antibodies, and (b) produce reticulum which is a special supportive stroma for lymph nodes and spleen; (4) some connective tissue cells in predisposed areas accumulate lipid and become fat cells thereby serving a storage function; and (5) connective tissues throughout the body constitute the principal means by which repair occurs. Mancini *et al.* (1962), recently injected fluorescent homologous albumin, globulins, and fibrinogen into adult rats and found that globulins and albumins accumulate in large quantities in connective tissues up to 8 hours, and begin to leave after 12 hours. On this basis, they postulate that connective tissues act as a pool for serum proteins.

Physically and physiochemically, connective tissues may be profitably viewed as immobile charged colloidal polyelectrolytes with properties that depend upon their composition which varies with anatomic sites, growth, age, and pathoses (Engel *et al.*, 1954; Catchpole *et al.*, 1956). Normal hydroxyproline/hexosamine ratios of 2.8 for cartilage, 12.2 for skin, and 30 for tendon illustrate the broad range of two major constituents of a few normal connective tissues (Engel *et al.*, 1960); these ratios are subject to change with the metabolic status of the involved cells which occurs with age and disease.

II. The Histochemistry of Connective Tissue Cells

A. Fibroblasts

Connective tissue cells are composed of a cytoplasm which contains ribonucleic acid demonstrable with basic dyes, and nuclei which contain deoxyribonucleic acid demonstrable also with basic dyes. However, the Feulgen stain is more specific for the identification of deoxyribonucleic acid (Lillie, 1954; Pearse, 1960). Supplemental proof for the type of the stained nucleic acid may be obtained by the digestion of sections with either ribonuclease or deoxyribonuclease (Lillie, 1954; Pearse, 1960). In nuclei there are small amounts of ribonucleic acids which are un-

detectable with general histochemical methods. Actively growing cells or productive cells display an increased ribonucleic acid content in their cytoplasms.

Basic nuclear proteins may be identified by staining acid-hydrolyzed sections preferably in a dilute solution of unmordanted hematoxylin in an alkaline buffer (Fullmer and Lillie, 1962), or with fast green FCF (Alfert and Geschwind, 1953). Zerlotti and Engel (1962) used 2,4-dinitrofluorobenzene without coupling for the demonstration of various protein groups, principally amino, sulfhydryl, and phenolic, and found that fibroblastic nuclei stained strongly. An enhanced color reaction of dinitrofluorobenzene-treated sections may be achieved by reduction, diazotization, and coupling with H-acid (Burstone, 1955). Weiss *et al.* (1954) noted that the nuclei of connective tissue cells stained intensely with the new method which they developed for the histochemical demonstration of protein-bound amino groups. They also noted that the cytoplasm stained. Connective tissue cells were found by Barrnett and Seligman (1958) to stain intensely with the new stain they developed for protein-bound α-acylamido carboxyl groups.

Glycogen and metachromatic material have been noted in the cytoplasms of fibroblasts. These inclusions are particularly prominent during growth and development (Laguesse, 1920; Bensley, 1934; Teilum, 1946; Gersh and Catchpole, 1949; and others).

Most normal fibroblasts probably have at least some alkaline phosphatase activity. Upon injury, alkaline phosphatase activity greatly increases. Raekallio (1961) made wounds in vital and in post-mortem skins in the backs of guinea pigs. He noted a greatly increased alkaline phosphatase activity of fibroblasts in the injured area within 8–16 hours after the injury. No increase of alkaline phosphatase occurred in wounds inflicted after death. Kawakatsu *et al.* (1960) reported an increased alkaline phosphatase of connective tissue cells of the gingiva in inflammation. Capillaries generally exhibit strong alkaline phosphatase activity; however, Scheen and Winkelmann (1961) describe the absence of alkaline phosphatase in capillaries in the skin of cats and lions.

Henrichsen (1956b) studied the calcification of tuberculous lymph nodes. He noted that fibroblasts undergoing degenerative changes manifested strong alkaline phosphatase activity. Calcification appeared to ensue concomitantly with necrosis of cells and the appearance of alkaline phosphatase.

Intense acid phosphatase activity has been noted in macrophages, and increased acid phosphatase activity has also been noted in inflamed fibroblasts by Raekallio (1961) and Fullmer (1964a,b). High acid phos-

phatase activity, which is presumed to be related to phagocytosis, has been noted in reticuloendothelial cells by many authors (Barka *et al.*, 1961). Some evidence exists for the concept that acid phosphatase and possibly other hydrolytic enzymes reside within a cytoplasmic component designated a lysosome by De Duve *et al.* (1955) and Novikoff *et al.* (1956). Nonspecific esterase has been noted in fibroblasts (Monis and Weinberg, 1961) although it is not as intense as in macrophages. The fibroblasts of tendon and sarcoplasm have also been observed to exhibit β-galactosidase (Schlager, 1959); the connective tissue cells and macrophages of human synovial membranes exhibit β-glucuronidase and *N*-acetyl-β-glucosaminidase (Pugh and Walker, 1961a,b). Mori and Kishiro (1961) found slight aminopeptidase activity in normal connective tissue cells of the gingiva, and an increased amount in inflamed gingiva. Monis *et al.* (1959), Raekallio (1961), and Okamoto *et al.* (1961) noted aminopeptidase activity in fibroblasts and increased activity in inflamed regions. Macrophages are also intensely stained for aminopeptidase in inflamed regions.

Fibroblasts and endothelial cells of the gingiva manifest intense lactic dehydrogenase, malic dehydrogenase, succinic dehydrogenase, DPN-diaphorase, TPN-diaphorase, and isocitric dehydrogenase (Fullmer, 1964a). Early investigations indicated that fibroblasts of the gingiva manifested a very weak α-glycerophosphate dehydrogenase (Fullmer, 1964a); however, subsequent investigations have revealed moderate staining of fibroblasts in sections incubated in the presence of menadione. Menadione is presumed to facilitate the transport of electrons.

Kawakatsu *et al.* (1960) also noted succinic dehydrogenase activity in fibroblasts and that the staining reaction was enhanced in inflamed tissues. Raekallio (1961) found an enhanced cytochrome oxidase activity in injured fibroblasts, and succinic dehydrogenase activity in connective tissue cells of the dermis of guinea pigs, with a slight intensification of activity 16 hours after injury.

B. Baker and Klapper (1961) demonstrated succinic dehydrogenase, cytochrome oxidase, malic dehydrogenase, lactic dehydrogenase, and DPN-diaphorase in foreign body giant cells that developed in response to implanted sponges.

Golarz *et al.* (1961) noted that proliferating connective tissue cells surrounding muscles in individuals with muscular dystrophy have enzymes that hydrolyze several high-energy phosphates and phosphorylated coenzymes, but do not affect phosphorylated glycolytic intermediates. On the basis of these results, they suggest that the concept of a primary connective tissue disorder in this disease should be entertained.

Monis *et al.* (1959) found intense aminopeptidase activity of sarcoma

cells in a fibrosarcoma of the leg. The edges of invasive tumors have been noted to display strong aminopeptidase activity (Burstone, 1956; Braun-Falco, 1957b), which prompted these authors to speculate that the capacity to tumors to invade was at least in part due to a proteolytic or peptidase action in the edge of these lesions. Monis *et al.* (1959) and others have demonstrated that intense aminopeptidase activity is a characteristic of young and growing connective tissues, and not a peculiar manifestation of these tissues adjacent to invading neoplasms.

Mottet (1961) implanted invasive carcinomas in rats and on the chorioallantoic membranes of chicks and thereafter determined histochemically the degree of aminopeptidase activity. He found an intense aminopeptidase activity in the connective tissue stroma surrounding carcinomas in rats not conditioned (irradiated), whereas activity was absent in conditioned rats. He concludes that aminopeptidase activity represents a host response to invading carcinomas.

Monis *et al.* (1960) examined the β-glucuronidase activity of several malignant neoplasms. They found either slight or no activity in lymphomas, a chondrosarcoma, and several other types of sarcoma.

Ogawa *et al.* (1961) cultured fibroblasts from embryonic chick hearts and noted cytoplasmic granules which stained with neutral red, for phospholipid, for acid and alkaline phosphatases, and for nonspecific esterase, whereupon they postulated that the granules are lysosomes. Ogawa and Shinonaga (1962) later demonstrated succinic dehydrogenase and DPN-diaphorase in chick embryo fibroblasts grown in tissue culture. D. Bloom *et al.* (1954) cultured fibroblasts and observed the effects of fixation, freezing and thawing, and freeze-drying under the phase contrast microscope. Henrichsen (1956a,b) also grew fibroblasts from chick embryo hearts; however, he was unable to demonstrate alkaline phosphatase in actively growing fibroblasts. He noted the appearance of alkaline phosphatase coincident with degeneration of the cells. Mancini *et al.* (1961a) grew fibroblasts from chick embryo hearts in tissue culture and added S^{35} periodically. The nature of the intracellular and intercellular substances produced was determined by stains for acid mucopolysaccharides, by testicular hyaluronidase digestion, and by barium hydroxide extraction. Radioactivity always appeared first intracellularly, within 1 hour after administration; after 3 hours, it appeared extracellularly in mucopolysaccharides. They were unable to detect acid mucopolysaccharides intracellularly.

Metabolic isotopic studies of connective tissue cells have been generally directed toward the elucidation of collagen and acid mucopolysaccharide production. This information has been heretofore best

obtained by autoradiographic studies conducted on detin, bone, and cartilage, and is described in Sections V and VI of this chapter.

B. Mast Cells

Mast cells were first described by Paul Ehrlich in 1879. At that time he called attention to the affinity of the granules in these cells for basic dyes. This characteristic still serves as the basis of our identification of mast cells. Mast cells are generally recognized by their cytoplasmic metachromasia. Many basic dyes have a metachromatic capacity; however, toluidine blue, azure A, azure B, and thionine are favorites today. Anionic groups in mast cell granules have the capacity to shift the absorption spectrum of certain basic dyes in dilute acidic solutions, resulting in a metachromatic effect. G. Bloom and Kelly (1960) utilize the strong anionic groups in mast cell granules for their staining method, which prescribes the treatment of sections in 0.1% solution of astrablau in approximately 0.7 N HCl at pH 0.2. In this case, nuclei generally fail to stain, and only some cartilages and mucins stain in addition to mast cell granules.

Mast cells are found in invertebrates as well as in vertebrates. They are frequently located in proximity to blood vessels and in the loose connective tissues. Tissue mast cells have been found to be numerous in the rat, dog, cat, bat, and calf, but fewer in rabbits and hares (Ehrlich, 1877; Westphal, 1891; Zimmerman, 1908). Mast cells develop late in intra-uterine medullary hematopoiesis (Ehrlich, 1877; Holmgren, 1946). Maximow (1910) and Alfejew (1924) have studied the development of mast cells in rats. Staemmler (1921) noted numerous mast cells in the developing thymus. Mast cells are generally not found in the central nervous system of man and most other animals, although they may be found in the meninges, about the choroid plexus, and about the pituitary stalk (Harris, 1900; Rosenheim, 1886; Tsusaki *et al.*, 1951; J. H. Gray, 1935). They may be found in the sheaths of peripheral nerves (Henschen, 1928).

Mast cells have been declared to derive from histiocytes (Downey, 1911; Herzog, 1916), from plasma cells (Downey, 1911; Sabrazes and Lafon, 1908), from lymphocytes (Audry, 1896; Sabrazes and Lafon, 1908; Downey, 1911; Michels, 1922), from fibroblasts (Bates, 1935), and from mesenchymal cells (Alfejew, 1924; Holmgren, 1946). Many subsequent authors have supported these contentions without absolute proof. Mitosis of mast cells has recently been observed (Hunt and Hunt, 1957; Asboe-Hansen and Levi, 1962). Deringer and Dunn (1947) noted that mitosis of mast cells occurs in fetal and neoplastic states.

Mast cells are easily degranulated. Fawcett (1955) injected water into