Enzyme Activities of Rat Connective Tissue Obtained from Subcutaneously Implanted Polyvinyl Sponge*

J. Frederick Woessner, Jr., and Robert J. Boucek

From the Howard Hughes Medical Institute, Miami, Florida

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A great deal of the current interest in the connective tissues has been focused on the extracellular components of these tissues, particularly collagen and the mucopolysaccharides. Alterations in the amounts and in the physical and chemical properties of these extracellular components have been implicated as contributing factors in many abnormal and diseased states such as rheumatoid arthritis, atherosclerosis, silicosis, the "collagen diseases," and aging. Changes in the amounts and properties of the extracellular substances are, in turn, ascribed to abnormalities in the metabolic processes of the connective tissue cells. Attempts to elucidate the nature of these abnormalities have been hindered by a lack of understanding of the normal metabolic processes of connective tissue. The chief barriers to such knowledge have been the low metabolic activities of most connective tissues and the difficulties in obtaining sizeable reproducible samples of relatively pure connective tissue amenable to study in vitro.

Recently, Boucek and Noble (2) have described a technique for stimulating the formation of new connective tissue by the subcutaneous implantation of polyvinyl alcohol sponge. This technique permits the simple and reproducible sampling of large amounts of connective tissue in a relatively pure state, as judged by histological examination (2). In the present study homogenates of such material have been quantitatively assayed for a variety of enzymes. The results indicate that this connective tissue possesses a wide range of enzymatic activities including representatives of the three major pathways of carbohydrate metabolism, a variety of esterases, and a unique pattern of proteolytic enzymes characteristic of all the other types of connective tissue thus far examined.

EXPERIMENTAL

Materials and Methods

Connective Tissue Samples—Three 100-mg squares of polyvinyl alcohol (Ivalon) sponge were implanted subcutaneously in the dorsal region of female Sprague-Dawley rats weighing 245 ± 40 g (2). The rats were maintained on Purina laboratory chow. After 24 to 27 days of development, the sponges were removed and freed from capsular tissue adhering to the surface. The three sponges were pooled, minced, and homogenized in 20 ml of ice-cold NaCl (0.9%) in the Virtis Homogenizer. The homogenate was filtered through gauze to remove sponge fragments and centrifuged for 15 minutes at 600 × g. An aliquot of the supernatant solution was further centrifuged for 30 minutes at 55,000 × g.

Determinations—Spectrophotometric measurements in the visible region of the spectrum were made with the Beckman model B spectrophotometer. Measurements in the ultraviolet region were performed with the Beckman model DU or DK-2 spectrophotometers. Nitrogen was estimated by the Conway microdiffusion method (3) after Kjeldahl digestion.

Enzyme Assays—The activity of each enzyme was determined with an average of eight preparations from the tissues of as many rats. In each assay system the conditions were such that the rate of reaction followed zero order kinetic expressions and the units of enzyme activity were directly proportional to the amount of enzyme added. This was assured by assaying each preparation at two different concentrations. In the case of the proteinase and phenolsulfatase it was necessary to construct a calibration curve to correct for deviations from linearity at higher enzyme levels. Glycylleucine dipeptidase, imidodipeptidase, and catalase followed first order kinetic expressions.

A unit of enzyme is defined as the amount of enzyme which catalyzes the conversion of 1 µmole of substrate per hour. Since there was a great amount of extracellular protein, including extravasated serum proteins, diluting the intracellular enzyme proteins of the homogenates, it was found that more consistent and meaningful results were obtained by relating the enzyme activities to the initial dry weight of the sponge before implantation, rather than to the protein concentrations. Therefore, the enzyme activities are expressed as units of enzyme per gram of sponge implanted. In the case of glycylleucine dipeptidase, imidodipeptidase, and catalase the first order rate constant, K, was measured in decimal logarithms and minutes. Enzyme activity for these enzymes is expressed as (K × final dilution of enzyme in the assay system × ml of enzyme per gram implanted sponge).

The temperature, method of enzyme preparation, and substrate concentration for each assay are given in Table I.

The liberation of p-nitrophenol from p-nitrophenylphosphate was used as a measure of the activities of acid (pH 4.8) and alkaline (pH 10.4) phosphatases (4, 5). The molecular extinction coefficient of p-nitrophenol in alkali at 410 µm was taken as 16.4 × 10³.

Aldolase was determined by the method of Sobel et al. (6) in which the product of the reaction, dihydroxyacetone phosphate, is caused to oxidize DPNH by the addition of catalytic amounts of α-glycerophosphate dehydrogenase. The molecular extinction
**Table I**  
Enzyme activities of connective tissue from sponges implanted in female rats for 24 to 27 days

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tissue preparation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Temperature</th>
<th>Substrate concentration&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Enzyme activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Carbohydrate metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldolase</td>
<td>2</td>
<td>23</td>
<td>0.1</td>
<td>718 ± 72&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Enolase</td>
<td>3</td>
<td>23</td>
<td>1.0</td>
<td>1,990 ± 359&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>3</td>
<td>23</td>
<td>0.33</td>
<td>5,880 ± 4,960&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Condensing enzyme</td>
<td>3</td>
<td>23</td>
<td>0.07</td>
<td>378 ± 93&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>1</td>
<td>38</td>
<td>20</td>
<td>586 ± 167&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>3</td>
<td>23</td>
<td>0.25</td>
<td>10,000 ± 2,000&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>2</td>
<td>23</td>
<td>0.66</td>
<td>790 ± 198&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6-Phosphogluconic dehydrogenase</td>
<td>2</td>
<td>23</td>
<td>0.66</td>
<td>506 ± 59&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>B. Proteolytic enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin C</td>
<td>2</td>
<td>37</td>
<td>50</td>
<td>401 ± 59&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proteinase, pH 3.2</td>
<td>3</td>
<td>37</td>
<td>(1.2%)</td>
<td>425 ± 135&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>2</td>
<td>37</td>
<td>50</td>
<td>4,010 ± 340&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycyglycine dipeptidase</td>
<td>2</td>
<td>37</td>
<td>50</td>
<td>16,000 ± 2,120&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycylleucyl dipeptidase</td>
<td>2</td>
<td>37</td>
<td>50</td>
<td>5.3 ± 2.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Imidodipeptidase</td>
<td>3</td>
<td>37</td>
<td>2.3</td>
<td>11.8 ± 3.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iminodipeptidase</td>
<td>3</td>
<td>37</td>
<td>2.3</td>
<td>23.7 ± 7.7&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>C. Esterases</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Acid phosphatase</td>
<td>2</td>
<td>37</td>
<td>6.33</td>
<td>852 ± 203&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>2</td>
<td>37</td>
<td>6.33</td>
<td>182 ± 62&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lipase</td>
<td>2</td>
<td>25</td>
<td>0.07</td>
<td>161 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nitrocatechol sulfatase</td>
<td>3</td>
<td>37</td>
<td>6.0</td>
<td>48.8 ± 9.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenolsulfatase</td>
<td>3</td>
<td>37</td>
<td>5.0</td>
<td>23.8 ± 5.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>D. Other enzymes</strong></td>
<td></td>
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<td></td>
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<tr>
<td>β-Glucuronidase</td>
<td>3</td>
<td>23</td>
<td>1.0</td>
<td>212 ± 43&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase</td>
<td>2</td>
<td>23</td>
<td>6.5</td>
<td>112 ± 29&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>2</td>
<td>23</td>
<td>0.18</td>
<td>979 ± 359&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytochrome c redoxase</td>
<td>2</td>
<td>23</td>
<td>0.018</td>
<td>100 ± 55&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutamic oxaloacetic transaminase</td>
<td>3</td>
<td>23</td>
<td>6.6</td>
<td>3,560 ± 870&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutamic semialdehyde reductase</td>
<td>2</td>
<td>23</td>
<td>1.0</td>
<td>200 ± 111&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Preparation 1 is the sponge-implant tissue homogenate filtered through gauze. Preparation 2 is the supernatant remaining after Preparation 1 has been centrifuged for 15 minutes at 600 X g. Preparation 3 is the supernatant remaining after Preparation 2 has been centrifuged for 30 minutes at 55,000 X g.

<sup>b</sup> In cases involving two substrates only the lower concentration is given.

<sup>c</sup> Standard deviation \( \sqrt{\frac{\sum x^2}{n-1}} \).

<sup>d</sup> Number of tissue specimens is given in parenthesis.

<sup>e</sup> These reactions followed first order rate expressions; activity is expressed as \( K \times \) enzyme dilution \( \times \) ml enzyme per g sponge implant.

The decrease in absorbancy at 240 μM served as a measure of \( H_2O_2 \) decomposition in the assay for catalase (9). The catalase activity was found to be markedly influenced by the small amounts of whole blood present in the tissue. The hemoglobin content of the sponge homogenates was determined by the method of Flink (10) and a correction was applied based on the average hemoglobin and catalase content of 10 samples of whole rat blood. In the determination of cathepsin C activity by its action on glycyl-γ-phenylalanine amide (11) aliquots were removed at intervals and assayed for liberated amide N by the modified Conway method of Schwert et al. (12). This same technique was employed in the case of leucine aminopeptidase acting on leucinamide, the assay conditions being those described by Smith (13). The assays for condensing enzyme (14),...
glucose 6-phosphate dehydrogenase (15), glutamic oxalacetic transaminase (16), lactic dehydrogenase (17), malic dehydrogenase (18), and 6-phosphogluconic dehydrogenase (19) were all conducted by standard methods based on changes in the absorbancy of the pyridine nucleotides at 340 μm.

DPNH-cytochrome c reductase was assayed by a modification suitable for crude preparations (20). The value of ε (reduced — oxidized) for cytochrome c was assumed to be 18.5 × 10⁶ at 550 μm (21).

The method of Sobel et al. (6) for enolase was modified by the addition of catalytic amounts of purified pyruvic kinase and lactic dehydrogenase. In this system phosphoglyceric acid was metabolized completely to lactic acid, resulting in the oxidation of DPNH which was measured spectrophotometrically at 340 μm.

Phenolphthalein glucuronide served as substrate for β-glucuronidase (22); the incubation time was reduced to 30 minutes and ε was taken as 22.8 × 10⁶ at 540 μm. The method developed by Smith and Greenberg (23) was employed for assaying glutamic semialdehyde reductase. Glutamic semialdehyde was synthesized from γ-γ-dicarboxy-γ-aminobutyraldehyde (24).

Glycylglycine dipeptidase and glycylleucine dipeptidase were assayed by the procedures of Smith (25) based on titration of the liberated carboxyl groups. Imidodipeptidase and iminodipeptidase were also measured in the assay system recommended by Smith (25) except that the substrate concentrations were reduced and the liberation of proline was followed by the colorimetric assay of Troll and Lindsley (26).

Lipase activity against p-nitrophenol acetate was determined by the liberation of p-nitrophenol (27). Under the conditions of this assay, the molecular extinction coefficient of p-nitrophenol was 10.3 × 10⁶ at 400 μm. Peroxidase caused the oxidation of 2,6-dichlorobenzenone-indo-3'-chlorophenol with a resultant increase in absorbancy at 645 μm (28), (ε = 24.4 × 10⁶). Succinic dehydrogenase was assayed by the manometric method of Kearney and Singer (29) with phenazine methosulfate as substrate. Proteinase activity against hemoglobin (30) was determined with 1.2% hemoglobin in 0.05 M citrate-phosphate buffer, pH 7.2. Activity was based on the μmoles of tyrosine liberated.

RESULTS AND DISCUSSION

The results of the enzymatic assays of extracts prepared from the sponge-induced connective tissues are presented in Table I. Approximate comparisons of these enzymatic activities with those reported for other tissues may be made by use of the finding that 1 g of implanted sponge gives rise to 11.9 ± 0.3 g wet weight of tissue containing 81 ± 6 mg of nitrogen soluble after centrifugation for 30 minutes at 55,000 × g.

Carbohydrate Metabolism—The three main pathways of carbohydrate metabolism all appear to be functioning in the sponge-implant connective tissue: anaerobic glycolysis (represented by aldolase, enolase, and lactic dehydrogenase); the tricarboxylic acid cycle (condensing enzyme, succinic dehydrogenase, and DPN-malic dehydrogenase); and the hexose monophosphate shunt (glucose 6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase). These pathways are represented by enzymes whose activities are all of a similar magnitude, except for lactic and malic dehydrogenases. For purposes of comparison it is found that the sponge-implant tissue contains (on a wet weight basis) about ⌜ the amount of lactic dehydrogenase, ⌝ the amount of malic dehydrogenase, ⌜ the amount of condensing enzyme reported on a wet weight basis for pig heart tissue (31-33). The α-glycerophosphate dehydrogenase is active in the sponge-implant tissue (34). In making these comparisons it must be borne in mind that the muscular tissues mentioned are among the most active sources known for these enzymes. Therefore, the sponge-implant tissue may be considered to be fairly active in its metabolism of carbohydrates.

An active carbohydrate metabolism would be anticipated in this connective tissue in view of the importance of carbohydrates and mucopolysaccharides as components of the ground substance. The question naturally arises as to whether enzymes directly involved in the synthesis of mucopolysaccharides can be demonstrated. Preliminary attempts to demonstrate hexosamine synthesis from glucose 6-phosphate and glutamine (35) and glucuronic acid formation from UDP-glucose (36) have not been successful.

There is no appreciable phosphorylase activity of the type found in liver or muscle (37, 38) indicating that the importance ascribed to glycogen for connective tissue formation in embryogenesis (39) probably does not apply to the sponge-implant tissue. The failure to detect α-glycerophosphate dehydrogenase activity by the method of Sobel et al. (6) was an unexpected result, since this enzyme had been reported to be active in cartilage (6).

Proteolytic Enzymes—Table I shows the unique pattern of proteolytic enzymes in the sponge-implant tissue. Only two major proteinase activities were found: one, an activity of the cathepsin C type and the other, an activity against hemoglobin with an optimum pH between 3.0 and 3.8 (see Fig. 1). This latter enzyme has not been further purified or characterized, but it appears to be related to the rat lung proteinase active at pH 3 to 4 described by Weiss (40) and further characterized as lung proteinase I by Dannenberg and Smith (41).

No activity could be demonstrated against hemoglobin at pH values greater than 6.0 (Fig. 1). Nor was it possible to detect activities of the type usually ascribed to trypsin (benzoyl-L-arginine ethyl ester, pH 8) (42), chymotrypsin (N-acetyl-L-tyrosine ethyl ester, pH 8) (42), carboxypeptidase A (carboxybenzoyl-L-glutamyl-L-tyrosine, pH 5) (43), and carboxypeptidase B (benzoyl-L-argininamide, pH 5) (12). Tissue homogenates were unable to liberate hydroxyproline-containing peptides from rat tail tendon collagen or oorein from elastin-orecin (44).

The sponge-implant tissue contains an active leucine aminopeptidase, but no appreciable carboxypeptidase activity (carboxybenzoyglycyl-L-phenylalanine, pH 7.5) (43). Aminopeptidase activity towards glycylglycylglycine was detected but was not determined quantitatively. The dipeptidases, glycylglycine dipeptidase, glyceylleucine dipeptidase, and imidodipeptidase (prolkase), were all quite active. But there was only slight activity of the iminodipeptidase (prolkase) type, and no activity against carnosine at pH 8 (25).

The glycylleucine dipeptidase appears to be related to the type found in uterine tissue as opposed to the type found in intestinal tissue. Adding zinc ions to the crude homogenates gave an activity of 5.3 compared to an activity of 1.5 when manganese ions were added (cf. Smith, 23)).

Imidodipeptidase possesses the ability to cleave the peptides glycyl-L-proline, and L-prolylhydroxy-L-proline (45) whereas iminodipeptidase cleaves peptides such as hydroxy-L-prolylglycine and L-prolylglycine (46). All of these peptide linkages are known...
to exist in collagen (47). It is conceivable that the presence of these enzymes bears a relation to the high rate of collagen turnover observed in the sponge-implant tissue (48).

The sponge-implant tissue has a distinctive pattern of proteolytic activity which bears a close resemblance to the patterns previously found by Fruton in rabbit skin and lung (49) and by Dannenberg and Smith (41) in rat lung. Both groups of workers found leucine aminopeptidase, aminotripeptidase, imidodipeptidase, and glycylglycine dipeptidase in the tissue investigated. Fruton also found glycyllleucine dipeptidase, and failed to find any activity against the usual substrates for trypsin, pepsin, and carboxypeptidase. Dannenberg and Smith (41) found that the lung contained iminodipeptidase and the proteolytic activity against hemoglobin at pH 4 mentioned above.

Est erases—The connective tissues exhibited hydrolytic activity towards a number of phosphate, sulfate, and acetate esters. The finding that the alkaline phosphatase activity of the soluble enzyme fraction is greatly exceeded by the acid phosphatase activity is of interest in light of the recent observations suggesting that alkaline phosphatase does not play an important role in collagen synthesis (50).

The hydrolysis of both p-nitrophenylsulfate and nitrocehol sulfate points to the presence of two types of arylsulfatase designated types 1 and 2, respectively, by Dodgson (8). This was confirmed by experiments in which 20 μmoles of KCN were added to the phenolsulfatase assay system and 15 μmoles of phosphate to the nitrocehol sulfate system. In both instances inhibition exceeded 90%, indicating the presence of two distinct enzymes behaving as the type 1 and 2 arylsulfatases. The phenolsulfatase activity (type 1) of this tissue differs from that of the rat liver in its ability to dissociate from the microsomes. The rat liver enzyme (sulfatase C) cannot be solubilized by homogenization in sucrose (61), but a considerable portion of the sponge-implant tissue activity is solubilized under the same conditions.

Other Enzymes—In the case of β-glucuronidase rough comparisons can be made with the results reported for sponge-implant tissue from guinea pigs (52). The rat tissue contains about 8 times as much β-glucuronidase as does the guinea pig tissue. These disparate findings emphasize the need for caution in attempting to extrapolate results from the tissues of one species to those of another.

An indication of the functioning of respiratory processes is given by the activity of DPN-cytochrome c reductase. It also appears that the metabolism of peroxides is an important process in connective tissue judging by the levels of catalase and peroxidase.

The amino acid of central importance in connective tissue metabolism is proline, which, together with its derivative hydroxyproline, constitutes about 30% of the collagen molecule. The possible formation of proline from glutamic acid has been indicated by the work of Smith and Greenberg (23) and Strecker (53). Glutamic semialdehyde reductase would be required in this interconversion and glutamic oxalacetic transaminase might also make a contribution. The activity of glutamic semialdehyde reductase in the sponge-implant tissue is about ½ the level found in rat liver (23), whereas the transaminase level is almost identical to that reported for human lung by Wróblewski and LaDue (54).

Advantages of Sponge-Implant Tissues—The majority of the enzyme activities reported here have been demonstrated previously in connective tissues such as bone, cartilage, and skin. However, the earlier studies have been chiefly histochemical and the more quantitative studies have often been ambiguous because of the presence of contaminating tissues such as gland cells, hair follicles, and muscle tissue. These problems are largely overcome by the use of sponge-implant tissue for quantitative studies; fibroblasts and their by-products represent the chief components, although the tissue still contains some small blood vessels, various types of blood cells, and extravasated blood proteins. In addition to the relative freedom from nonconnective tissue components, the sponge-implant tissue also offers the advantage of ease and uniformity of sampling and the possibility of repeated testing without killing the experimental animal. It is felt that the sponge-implant tissue gives a truer picture of the metabolic activities of connective tissue than would tissue cultures because of the tendency of cultured cells to undergo marked alterations in enzyme activities (55).

The sponge-implant tissue has been employed in a quantitative study of the alterations in enzyme activities during the period of connective tissue development from the time of sponge implantation to maturity. The results of this study will be the subject of a subsequent report.

**SUMMARY**

1. The levels of the activity of 26 different enzymes have been studied in connective tissue obtained from female rats by the sponge-implantation technique.
2. Enzymes representative of the pathways of anaerobic glycolysis, the tricarboxylic acid cycle, and the hexosemonophosphate shunt are present and have activities of a similar magnitude.
3. The proteolytic enzymes present include glycglycine dipeptidase, glycyllleucine dipeptidase, imidodipeptidase, iminodipeptidase, aminotripeptidase, leucine aminopeptidase, cathepsin C, and a proteinase active toward hemoglobin with an optimum activity in the pH range 3.0 to 3.8. This pattern of activities is quite similar to that found in rat skin and lung.
4. The sponge implantation technique provides samples of actively metabolizing connective tissue suitable for a variety of metabolic studies.
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