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Introduction

The matrix of certain mammalian tissues contains several types of sulfated and non-sulfated mucopolysaccharides (cf. Meyer, 1948) in addition to other carbohydrates (cf. Glegg et al, 1954), cement substance and fibrils.

One of the non-sulfated mucopolysaccharides, i e., hyaluronic acid, has been demonstrated in connective tissue, in teeth, in brain tissue, in the intervertebral discs, bovine vitreous humor, synovial fluid, the lung and other tissue. (Bairati et al, 1952; Bensley, 1950; Friedman, 1953; Holland, 1954; and others).

The mucopolysccharides are depolymerized under certain conditions by specific enzymes; thus hyaluronic acid is depolymerized by the "spreading factor" (Duran-Reynals, 1928), the "Eizytoplasma auflosende Substanz" (Yamane, 1935), the "sperm enzyme" (Pincus and Enzmann, 1936). Chain and Duthie, 1940, have claimed that all these substances are identical and have renamed them "hyaluronidase."

The systems of mucopolysaccharides and their enzymes may be of importance in the pathogenesis of several diseases, notably those termed "collagen diseases" by Klemperer (1950). The most important of these diseases is rheumatic fever, which leads to lesions in the various parts of the body, especially the cardiovascular system. Coburn (1930) has made a connection between the hyaluronidase-producing Streptococcus hemolyticus, group A, and rheumatic heart disease. Several other workers have shown that lesions similar to those occuring in human rheumatic hearts may be produced by repeated infections of animals with the suspected organism (Fortune, 1953; Miale, 1951; Weaver, 1951; Glaser, 1951; Murphy and Swift, 1949, and others.)

If the mucopolysaccharides of the ground substance are in any way implicated in the genesis of lesions in the rheumatic heart, the study of these substances becomes a matter of great importance. As a preliminary step towards such studies we have attempted to trace the distribution of acid mucopolysaccharides in various parts of the hearts of human embryos and fetuses of various ages. In a later report we shall present the results of similar experiments on the hearts of children, adults and aging persons.

Materials and Methods

The material for the present series of tests consisted of twenty human embryos and fetuses of various ages, as shown in the appended table (I). The material came from the Anatomical Museum of Still College; some of the specimens were sent in by practicing physicians to whom we express our gratitude. The smaller specimens were embedded in paraffin and sectioned in toto; the

Table I

Material used and preliminary treatment.

Specimen number	CR (or T) length and estimated age	Fixation and sections
1	T 25 mm, 52 days.	formalin, entire heart
2	CR 42 mm, 84 days.	formalin, entire heart.
3	CR 40 mm, 70 days.	formalin, entire heart.
4	CR 50 mm, 77 days.	formalin, entire heart.
5	CR 100 mm, 105 days.	formalin, entire heart.
6	CR 80 mm, 91 days.	formalin, entire heart.
7	T 15 mm, 40 days.	formalin, entire embryo.
8	CR 35 mm, 65 days.	formalin, entire heart.
9	CR 110 mm, 108 days.	area of A-V valves, Bouins.
10	CR 75 mm, 84 days.	formalin, entire heart.
11	CR 50 mm, 80 days.	formalin, entire heart.
12	CR 45 mm, 76 days.	formalin, entire heart.
13	T 26 mm, 26 days.	formalin, entire embryo
14	T 30 mm, 63 days.	formalin, entire embryo.
15	CR 36 mm, 250 days.	Bouins, heart valves.
16	CR 180 mm, 180 days.	Bouins, heart valves.
17	T 9.8 mm, 33 days.	formalin, entire embryo.
18	CR 40 mm, 70 days.	formalin, entire heart,
19	CR 8 mm, 36 days.	formalin, entire embryo.
20	CR 20 mm, 50 days.	formalin, entire heart.

larger specimens were dissected and only the heart was embedded, while the largest fetuses were dissected and only portions of their hearts were used.

The occurrence and distribution of acid mucopolysaccharides in various parts of the hearts was tested histochemically (cf. Lillie, p. 291) by means of metachromatic stains on deparaffinized sections. In other series of tests, sections of the same hearts were subjected to digestion in buffered hyaluronidase, after which they were stained under identical conditions as were the untreated sections. In a third series, heart sections were tested for metachromasia after treatment with boiled enzyme.

In a preliminary survey, the following metachromatic stains have been listed and their color changes recorded (Table II).

Table II

Metachromatic stains and their color changes in areolar connective tissue.

Stain used	Color change
Methyl green pyronin	red——yellow
Bismark brown**	yellow——brown
Azur II*	blue——purple
Thionin**	blue——rose
Toluidin blue	blue——rose
Azur C (dimethyl)**	blue——purple
Azur A (trimethyl)**	blue——purple——green
Azur B eosinate (tetramethyl)**	blue——purple
Safranin O	yellow——orange
cresyl violet	violet——reddish

* Obtained from Coleman and Bell Co., Norwood, Ohio.
**Obtained from Allied Chemical and Dye Corp.
The remaining ones obtained from Gruebler Co.

The deparaffined sections were affixed to glass slides by means of a dilute solution of collodion in ether; the solution must be very dilute otherwise it delays the penetration of the enzyme into the tissue. The film of collodion will not interfere with the staining reaction.

The best results were obtained with the stains toluidin and thionin in concentrations of 0.0027 per cent, acting on the tissues for five minutes. The stains were made up in distilled water buffered with acetates to the pH values of 2.7, 3.7, 4.7, 5.7 and 6.7. (Wal-

pole, cf. Lillie, p. 450).

The metachromatic reaction proceeds very fast and the slides must be examined within 2-5 minutes after removal from the staining jars. The stains leach out in water and even quicker in the water-alcohol mixtures; however, permanent slides showing some metachromasia can be prepared by air-drying the sections before clearing and embedding. The enzymatic digestion was done by immersing the deparaffined slides for 24 hours in a solution of hyaluronidase in distilled water, buffered to pH 6.4.*)

Results

The acid mucopolysaccharides change the color of toluidin and thionin to various shades ranging from purple to rose, depending on such factors as the presumable proportion of sulfated to non-sulfated polysaccharides and the pH of the staining solution. This enables the observer to differentiate between sulfated and non-sulfated components to some extent: thus the neutral mucopolysaccharides fail to stain below pH 6.5, hyaluronic acid does not stain below pH 4.5, while chondroitin sulfuric acid shows no staining below pH 2.0 (cf. Greep, p. 39).

The best staining reaction was observed in the connective tissue of the coronary sulcus, but metachromasia appears also in various degrees in the following structures: the heart cushion, interventricular septum, A-V valves, pericardium, endocardium, and tissues just below these linings. The walls of the aorta and pulmonary artery also show pronounced metachromasia.

The intensity of the reaction varies with the pH of the staining solution and possibly with the age of the embryo or fetus from which the specimens were obtained. There seems to be a slight decrease of the intensity of the matchromasia with increasing age, but the differences are slight and perhaps doubtful. (Table III). (Holmgreen, 1939, has reported that metachromasia decreases with age.)

The effect of pH on the intensity of the staining reaction is more pronounced and unmistakable. The staining diminishes noticeably with the pH shift to the acid side and practically ceases at pH 2.0; it is well-marked between pH 2 and pH 4.7 and reaches a maximum with pH 6.7, the highest value listed. (Table IV).

The piling-up of stain at the edges of tissue sections, notably at the pericardium, endocardium, between the fibres of the cardiac muscle and the great vessels of the heart presents a perplexing phenomenom. This accumulation of stain along edges seems to be a physical process similar to the formation of ice crystals around dust nuclei on a windowpane.

Treatment with hyaluronidase in order to remove the hyaluronic

*The enzyme preparation, lyophilized hydase, was purchased from Wyeth, Inc., Philadelphia.

Table III

Strength of the metachromatic reaction observed in the connective tissue of the coronary sulcus in sections of hearts of various ages.

Serial number of specimen	Age of individual	Strength of metachromasia*, untreated hearts	Strength of metachromasia, hy-ase treated.
13	26 days	***	**
4	50 days	***	**
3	70 days	*	*
5	105 days	**	*
16	180 days	*	*
15	250 days	*	°

The strength of metachromasia was judged on an arbitrary scale.

° Denotes no staining.

*** Denotes intensity of stain in tracheal cartilage sometimes included in sections. (Maximum staining).

Table IV.

Strength of the metachromatic reaction obtained by staining heart sections at various pH values.

Serial number of specimen	pH values 20	2.7	3.7	4.7	5.7	6.7
7 (40 days)	°	*	*	**	***	***
9 (108 days)	°	*	*	**	***	***
16 (180 days)	°	°?	*	*	**	**

acid did not abolish metachromasia but decreased it slightly (control sections cut as frozen sections showed the same staining reactions as deparaffined slides).

The enzymatic digestion as well as the pH effects seem to indicate that the metachromasia in these hearts may be due to the presence of several acid mucopolysaccharides, only one of which is removed by the action of hyaluronidase. The relative amounts of sulfated and non-sulfated mucopolysaccharides cannot be ascertained from the present experiments until it is known how much of each has been removed by the preliminary treatment with fixatives and alcohol-water mixtures.

Experiments in which deparaffinized heart sections were treated with hyaluronic acid which had been boiled for ten minutes in 0.01 M acetic acid gave the following results: At pH 6.5 and pH 4.7 the enzyme treated sections stained as well as the untreated controls;

at pH 2.7 the treated sections showed less metachromasia than the controls.

The present experiments show the presence of metachromatically staining substances in the hearts of human fetuses and embryos of various ages. Earlier workers have claimed that the heart contains hyaluronic acid (Holmgreen for human hearts, Deis and Leon for bovine hearts); Meyer* stated that "the occurrence of hyaluronic acid in the human heart is only presumptive."

Our experiments fail to distinguish clearly between the sulfated and non-sulfated mucopolysaccharides, both of which produce metachromasia. Since our hearts show metachromasia in the pH staining range between 2 and 4.7, it must be assumed that the staining reaction in this area is due to the presence of chondroitin sulfuric acid or other substances, giving the same reaction.

Lillie states that boiling for ten minutes in 0.1 M acetic acid destroys hyaluronic acid and amylase, but fails to destroy the chodromucinases. The metachromasia of the umbilical cord, nucleus pulposus and cartilage are abolished by both raw and boiled testicuar "Hyaluronidase," which leads him to conclude that the metachromatic substances in these structures are chondromucins. Lillie holds that "at present it is not clear whether we possess a chemical method for localizing hyaluronic acid and chondromucins."

On the other hand, our experiments show a distinct decrease of metachromasia below pH 4.7 which seems to indicate that hyaluronic acid is present in the heart sections.

Summary and Abstract

Sections of hearts of human embryos and fetuses were tested by means of metachromatic stains for the presence of acid mucopolysaccharides. All sections gave positive results and the intensity of the staining reaction decreased with decreasing of pH of the staining and to a very slight degree with increasing age of the fetuses.

The histochemical tests indicate the presence of hyaluronic acid as well as chondroitin sulfuric acid and possibly other substances capable of metachromasia.

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