Aging of Collagen in Complex Tissues. A Micromethodological Study of the Thermal Reaction[1]

The thermal and chemical reactivities of collagen have often been used as age tests (for review see e.g. VIIDIK[2]), the generally accepted concept being that this phenomenon is a phase transition involving melting of crystalline regions[3]. In parallel-fibred samples, the isotonic shrinkage[4] or isometric tension development[5] are easily assessed. As collagen matures it is increasingly cross-linked. That this would result in an elevation of the melting temperature was predicted from a theoretical point of view[6] and subsequently demonstrated experimentally[7-9]. On the basis of these observations, the present method was developed for the evaluation of the maturity of collagen in complicated patterns.

Theory of method. As the basic unit, a Leitz Ortholux microscope was used. For the generation of heat, Leitz heating and cooling microscope stage '80' was used in conjunction with a variable transformer to ensure a proper heating speed. To avoid heat dissipation a special chamber was built for the specimen. A hole with a diameter of 9 mm was drilled through a brass plate measuring $25 \times 76 \times 6$ mm. To get a translucent bottom to the chamber, the brass plate was glued with Araldite to a 2 mm thick glass plate of the same size. Thus a circular chamber, 9 mm in diameter and 6 mm deep, was achieved. The specimen could be fixed to the bottom of the chamber by securing it between a circular spring and the angle between the wall of the chamber and its bottom or by placing a glass disc on top of it. In the former case, no shortening of the specimen could occur and in the latter case it was free to move horizontally. The chamber with a specimen secured in the latter way is pictured schematically in Figure 1.

Although the heating and cooling microscope stage was equipped with a thermometer in its periphery, an Araldite embedded thermistor was immersed into the fluid and mounted with its measuring point just above the specimen in the chamber to ensure accurate temperature readings from the immediate milieu of the specimen (Figure 1). A bridge circuit was used to read the resistance change in the thermistor and thus the temperature. The conduction of the heat in the system proved well reproducible from one run to another.

The recording of the thermal reactivity experiments was performed in a polarizing system with crossed polarizers and a lambda filter. An objective 3.5/0.10 was used in the Ortholux microscope and microphotographs were taken with a Leica MDa camera on a microadapter at certain temperature levels. Flash illumination (1/1000 sec) from a Braun F-800 coupled to a F-650 straight discharge tube adapted to the optics of a Leitz microflash unit was used for exposure. As the lambda filter converts the birefringent and non-birefringent areas into different colours, yellow or green for birefringent elements depending on the plane of the light waves, and red for non-bifringent ones. Agfacolor CT 18 colour film was used for most experiments giving good reproduction of the original colours.

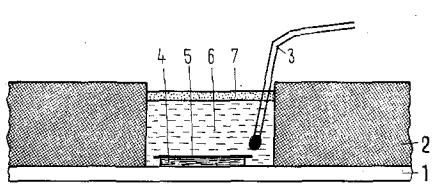

Fig. 1. Schematic picture of the test chamber sectioned through the middle. 1. the bottom glass plate; 2. brass block; 3. thermistor; 4. specimen; 5. glass plate to keep the specimen even; 6. Ringer's solution and 7. liquid paraffin.

The method requires that the specimen can be viewed in transmitted light microscopy. Rat tail tendon fibers are thin enough to transmit light but most other tissues must be sectioned. For the experiments discussed below, the tissues were cut into 25 µm thick slices in a freezing microtome with rapid freezing and thawing. They were then kept in a buffered Ringer's solution (pH 7.4) until mounting into the chamber, then containing a small amount of Ringer's solution on its bottom. The slice was either fixed by the circular spring or by the glass disc. Thereafter the chamber was filled with Ringer's solution and evaporation from the surface was prevented by a thin film of liquid paraffin. This is shown schematically in Figure 1.

A collagen fiber denatures practically simultaneously throughout its length, as can be demonstrated on tail tendon fibers. Already a freezing mictorome section of skin, displaying a two-dimensional picture of a three-dimensional mesh-work, exhibits a multitude of fibers denaturing at small temperature intervals. Therefore, from viewing the series of microphotographs, it was impossible to tell the exact point where denaturing occurs. This task became even more impossible for specimens with collagen of varying degrees of maturity. Therefore, the recordings from an experiment are best analysed frame by frame. The 24×36 mm frame was divided by superimposing a grid on the frame with horizontal and vertical lines into square compartment, the size and amount of which were depending on the type of specimen. Then the squares where birefringence was exhibited were counted in a low power microscope. Only the green variety was counted as transitions from yellow to red are less easy to determine. In control countings no decisive differences could be found between counts of 1. green, 2. yellow, and 3. green and yellow. Repeated counting proved most reproducible for green. The amount of squares with birefringence at 20 °C was set as 0% denaturation and when no birefringence could be seen in any frame was set to 100%. This gradual decrease of birefringence, i.e. more and more fibers are denatured, is examplified in Figure 2, which demonstrates the calculations from the counting of two experiments.

In a more complicated specimen, as a healing wound, the various areas are best drawn on the grid, and the different areas are calculated individually. As the specimen shrinks when the critical temperature is reached, it may be necessary to draw outlines on several grids for the same specimen.

The environment of the specimen during preparation and the testing conditions are of great importance. If a tendon fiber is allowed to dry before testing and resoaking is attempted in Ringer's solution, the result differs from those experiments performed on fresh tissue. Another

[1] This work was supported by Swedish Medical Research Council (Project No. B70-12X-2883-01) and by Carin Trygger Memorial Fund through the Swedish Society of Medical Sciences and performed at the Department of Anatomy, University of Gothenburg, Sweden.

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feature is demonstrated by Figure 2, which shows thermal reaction in the skin collagen of an eight month old rat. The curve with solid circle shows the loss of birefringence in a specimen mounted under a glass disc (isotonic), while the curve with open circles shows the spring mounting (fairly isometric). It is evident from the figure that keeping the specimen in isometric conditions elevates the denaturing temperature registered. This is in agreement with the investigations of FRENKELJ et al.[10] on tail tendon fibers from 6-month-old rats. They found that the denaturing temperature could be elevated somewhat if constant loads on the parallel-fibred collagen structure in hydrothermal experiments was increased. At the same time they found that the magnitude of shortening was diminished. This can be explained by the fact that denaturing means transition from an orderly state to one, in which the molecules are randomly coiled. This transition is to a certain limit prevented by imposing a more strict orientation through tension. From this it must be evident that it is not safe to draw conclusions from results of tests, where the specimen has been subjected to unknown loads during isometric testing.

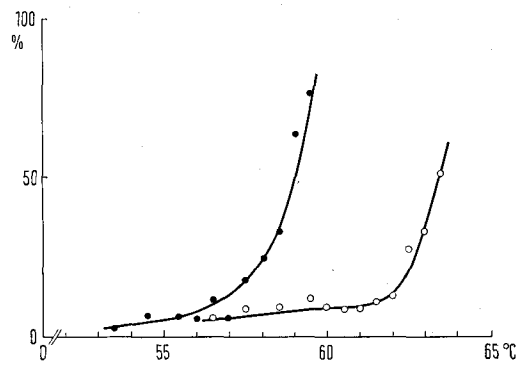

Fig. 2. Percent of initial birefringence lost with increasing temperature. Mean value of experiments on skin from an 8-month-old rat, isotonic (solid circles), and isometric (open circles) test, performed in Ringer's solution buffered to pH 7.4.

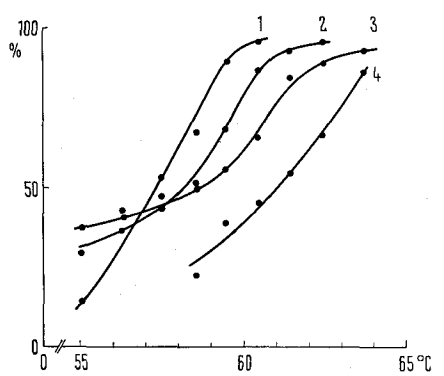

Fig. 3. Percent of initial birefringence lost with increasing temperature. Mean values of isotonic tests on human skin specimens: 1. fetus 6 months: 2. 2 months; 3. 6 years and 4. 55 years.

Discussion on the applicability of the method. To evaluate the thermal reactivity characteristics of parallel-fibred tissue, especially if the amount of it per unit specimen length is easily estimated or standardized, the isometric or isotonic method at a standard temperature is the method of choice. However, with complicated patterns of fiber arrangement or when fibers of various degrees of maturity are present, the method presented here offers advantages.

Figure 3 shows evaluation of the thermal reactivity of human skin of various ages. It seems that the thermal denaturation occurs more abruptly in the older individual than in the young one, i.e. the collagenous elements in the young one are of somewhat varying denaturing temperatures, some of which are considerably lower than those of the older skin while some of them do not differ very much. This is the same phenomenon as can be demonstrated chemically with the varying extractability of collagen (Cf. e.g. KLEIN et al.[11]).

In a healing wound, where collagens of considerably different degrees of maturation are present, the wound reaction area itself, adjacent skin and collagen distant to the site of trauma can be assessed separately (HOLM-PEDERSEN and VIIDIK[12]). It has been claimed (DOUGLAS[13]) that wound collagen has physical properties other than ordinary dermal collagen on the ground that wound collagen fails to show birefringence although it is indistinguishable from ordinary collagen by histological staining. With the methodology presented in this paper, it is shown that this 'phenomenon' is an artifact caused by the combination of histotechnique which for paraffin embedding includes heating and low denaturing temperature of young collagen. This collagen, in freezing microtome sections, exhibits the same type of birefringence as ordinary skin collagen but loses it at a considerably lower temperature than the collagen of adjacent skin, which is more mature.

Zusammenfassung. Die Messung des Denaturationsdunktes als Parameter für die thermale Reaktion des Kollagens in geometrisch komplexen Geweben oder von Kollagenen verschiedener Reifegrade wird beschrieben und Beispiele im Bereich von Altersunterschieden und Wundheilung werden gegeben.

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[14] The technical assistance of Miss ULLA-BRITT LINDGREN in the development of this method is gratefully acknowledged.

Electrophoretic Analysis and Molecular Weight Estimation of Proteins from Guinea-Pig Brain Subcellular Fractions

The isolation and characterization of the chemical components of the synaptic membranes is a prerequisite toward a complete understanding of the complex events that take place at the synaptic level. Techniques for isolation of brain subcellular particles such as 'synatosomes' have been available in the last decade[1,2] and many comparative studies have been carried out on the different fractions obtained by gradient centrifugation.