Thermal Effects on Birefringence of Tendon with Aging[1] (34451)

M. B. ENGEL AND H. R. CATCHPOLE

Departments of Orthodontics, Histology, and Pathology, University of Illinois at the Medical Center, Chicago, Illinois 60612

The maturation of collagenous tissues is accompanied by chemical and physical changes. Rat tail tendon provides a particularly good example because it is a relatively homogeneous connective tissue consisting of collagen predominantly with some ground substance and a relatively low concentration of cells.

Verzar has extensively studied the effects of thermal change on physical and chemical properties of this tissue (1). The shrinkage temperature of mammalian collagen and of tendon is usually considered to be in the neighborhood of 62°. However, changes in tension and the release of soluble materials are known to occur at lower temperatures. These effects vary with age.

Tendon has a highly oriented structure and appears positively birefringent when viewed with the polarizing microscope (2–4) (Fig. 1). At the shrinkage temperature birefringence is lost. We report on the effects of graduated thermal change on this birefrigence with respect to growth and maturation.

Materials and Methods. Male Sprague-Dawley rats were obtained from the Simonsen Laboratories. Five age groups with three or four animals in each were selected: 1, 2.5, 3.5, 7.5, and 24 months. The animals were anesthetized with ether and the skin over the middle third of the tail was incised and the tendon was exposed. Approximately 2-mm sections of tendon were excised and attached to small brass blocks with a drop of blood. The blood, with affixed tissue, was then rapidly frozen in isopentane chilled in liquid nitrogen at —150°. The blocks were transferred to a Dittes cryostat without thawing and mounted in the microtome. The ambient temperature was maintained at —40 to 60°. Longitudinal sections were cut with the microtome set at 8 μ. These were transferred to tissue holders at —40 to —60° and then to a freeze–dry chamber also mounted in the cryostat. Tissues were dehydrated *in vacuo* at —40° for at least 24 hr.

For examination with the polarized light microscope, sections were mounted on slides in rat serum ($n\mathrm{D}_{25} = 1.355$). After the cover glass was applied, it was sealed with petroleum jelly. A Leitz polarizing microscope equipped with a heating stage was employed. Observations were made with a 10× objective and an 8× eyepiece using an adjustable diaphragm. A relatively small field (diam ~ 150 μ) containing few cells was selected to minimize effects of thickness and heterogeneity in the preparations. The tissue was brought to the position of maximum extinction between crossed polars. The stage was then rotated through 45°, bringing the field to maximum brightness. The stage was maintained at 37° during this procedure. Near monochromatic illumination was provided by using a narrow band interference filter with a peak at 550 mμ.

Light transmission through the tissue was measured with a Photovolt (520 M) photometer used in conjunction with a photocell. Final adjustments for maximum brightness were aided by checking the photometer as the stage was rotated. For the initial reading the instrument was set at 100% transmission. The specimen temperature was monitored by placing a Yellow Spring thermistor probe in contact with the slide. The temperature was then raised 1°/min over the range 37–62° and the corresponding optical density readings were recorded. In each age group 10–15

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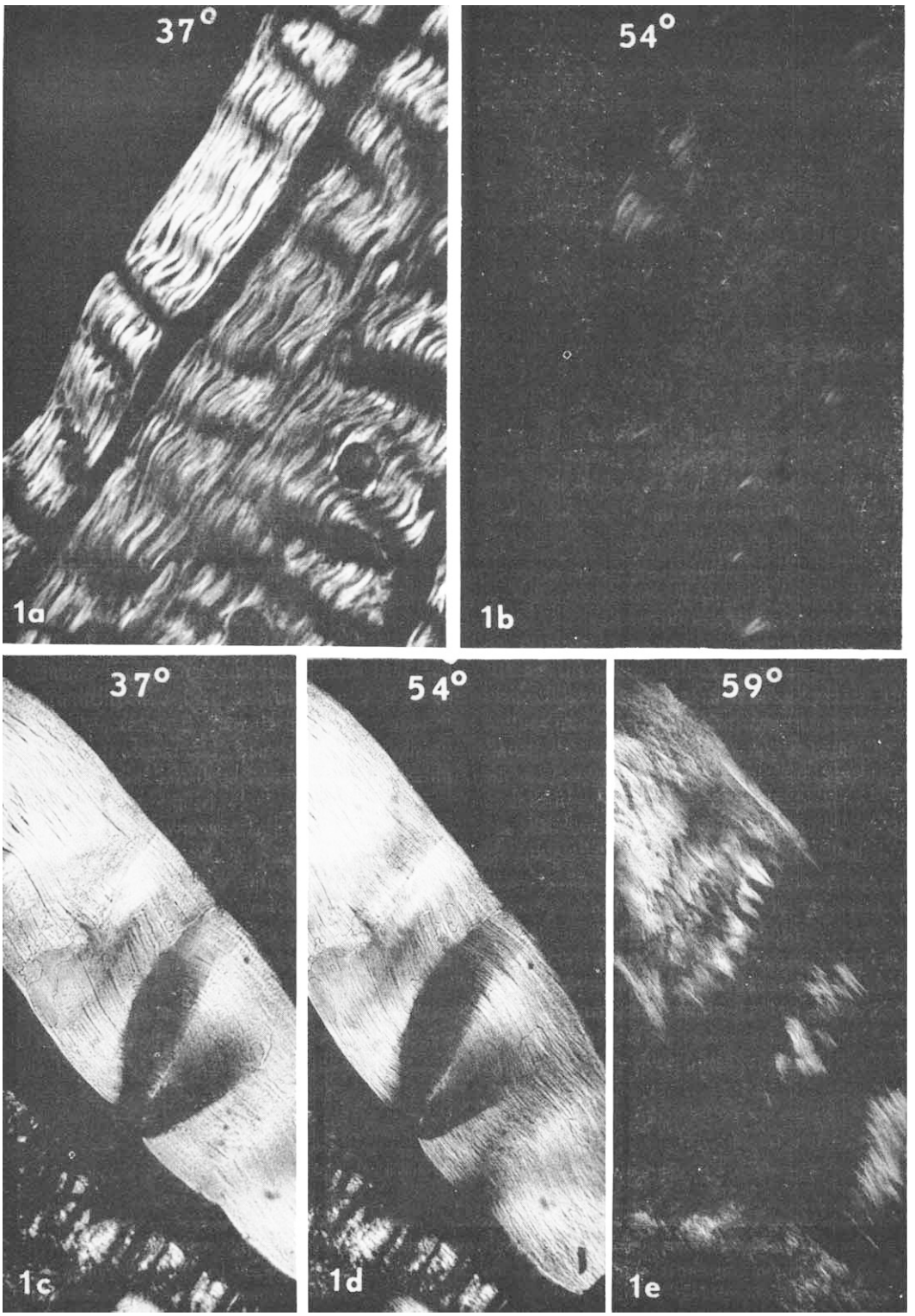

FIG. 1. Photomicrographs of rat tail tendon viewed with the polarizing microscope. Crossed polars with tendon aligned in position of maximum brightness at 45°. Temperatures are indicated in degrees centigrade. (a) tendon from 1.5-month-old rat; (b) tendon from 1.5-month-old rat; the tissue is almost completely isotropic at 54°; (c) tendon from 24-month-old rat; (d) tendon from 24-month-old rat; little change is seen at 54°; and (e) tendon from 24 month-old rat; at 59° birefringence is disappearing.

pooled samples were studied. Mean values were determined together with standard deviations and standard errors. Significance of the differences between age groups was calculated at six temperatures between 50 and 60°. For each age group a curve was plotted to show the relation between temperature and optical density (Fig. 2).

Results. When sections of young, mature, and aged tendon were compared using the polarizing microscope, certain qualitative differences were apparent. In young tissues the fibers were loosely arranged and cells were more numerous and larger than in the other two groups. With maturation and aging the tendon became more compact and cells were flattened. It appeared that the birefringence was higher in mature and aged animals, but measurements of retardation were not made.

Anistropy with no loss of birefringence was noted over the range 37–48° (Fig. 1a, c). Minor fluctations and slight increases in brightness appeared to be related to initial swelling. In the 1–2.5-month-old animals, greater losses in birefringence were observed at lower temperatures and the rate of change was accelerated so that isotropy was attained at around 54° (Fig. 1b). At 3.5 months the optical density temperature curve was shifted to the right with respect to temperature by about 4° as compared to the 1-month tendon. Beyond this age and up to 24 months further change was limited (Fig. 1). Over the age range studied there is a 6° difference in temperature required to abolish birefringence, *i.e.*, 54–60° (Figs. 2 and 1b, d, e). Results and statistical evaluations are given in Table I.

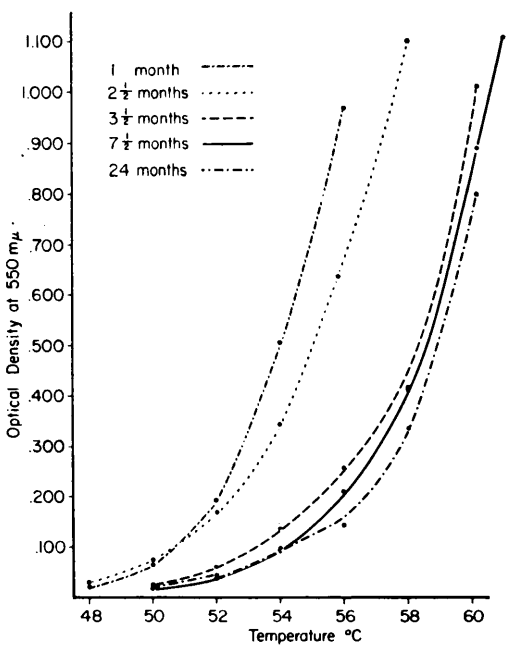

FIG. 2. Birefringence changes in rat tail tendon: Graph showing relation between birefringence as measured by optical density readings and temperature in tendon from rats of different ages.

The results bear a relation to reported shrinkage temperatures for collagen and for tendon (5). In some respects they are more revealing since they show that changes in micellar structure, *e.g.*, increases in disorder, are not abrupt but in the initial phases rather gradual. Curves of the data are empirical since it became apparent in the early stages of the work that the rate at which the temperature was elevated influenced the curves. The contraction temperature of collagen shows a similar relationship (6). Consequently, this variable was carefully standardized at 1°/min.

With respect to thermal effects on birefringence, the major changes in tendon occur within approximately the first 3.5 months. At 3.5 months, the animals have reached maturity. Subsequently, through 7.5-24 months the changes, while clearly in the same direction, are of lesser magnitude and significance. The degree of significance of the changes with age tends to increase as the maintained temperature approaches the "melting point" where anisotropy is completely lost.

Discussion. Since the tendon consists of collagen and other matrix substances, including a ground substance comprised of glycosaminoglycans and glycoproteins, these changes cannot be attributed solely to one component. The experimental arrangement, employing a physiologic ambient medium with a fairly low refractive index, was calculated to reveal total birefringence, *i.e.*, both form and intrinsic birefringence attributable to the whole structure. Thus the aging changes observed are not strictly comparable to those observed (or to the lack of them) in extract-

TABLE I. Optical Densities of Rat Tail Tendon of Different Ages at Maximal Birefringence.[a]

Temp (0°)	Months				
	1	2.5	3.5	7.5	24
48	0.020 ± 0.002	0.029 ± 0.005	Remained anisotropic	Remained anisotropic	Remained anisotropic
50	0.064 ± 0.011	0.069 ± 0.013	0.024 ± 0.005[b]	0.014 ± 0.003	0.017 ± 0.004
52	0.180 ± 0.007	0.166 ± 0.027	0.057 ± 0.010[b]	0.035 ± 0.003	0.040 ± 0.010
54	0.506 ± 0.050	0.343 ± 0.044[b]	0.134 ± 0.020[b]	0.094 ± 0.020	0.095 ± 0.016
56	0.970 ± 0.073	0.631 ± 0.061[b]	0.256 ± 0.035[b]	0.210 ± 0.024	0.140 ± 0.033[c]
58	—	1.100 ± 0.100	0.417 ± 0.053[b]	0.412 ± 0.027	0.328 ± 0.085
60	—	—	1.010 ± 0.177	0.885 ± 0.065	0.800 ± 0.134
61	—	—	—	1.111 ± 0.113	—

[a] Means ± standard errors.

[b] Significantly different ($p \leq 0.01$) from preceding month.

[c] Significantly different from 3.5-month value.

ed and purified collagen by Salazar *et al.* (7). However, in thermal disruption of collagen there is evidence of solubilization of hydroxyproline-containing residues and increasing amounts of soluble carbohydrate including hexosamine (1, 8). It is probable that labile electrostatic and other low energy bonds are broken initially, followed by disruption of higher energy bonds at the higher temperatures. More extensive cross-linking of mature and aging tendon would account for the increased resistance to this kind of change observed in the older animals.

Aside from the relevance of these results to questions of physiologic maturation and aging the method offers other uses. For example, in various physiologic and pathologic conditions where the state of connective tissue is modified, application of polarization microscopy could provide a sensitive test of a change in organization.

Summary. The effects of thermal changes on the organization of connective tissue of rat tail tendon was studied by polarization microscopy. Loss of birefringence in the temperature range 48–60° occurs most rapidly in young animals (1–3.5 months). After 3.5 months the trend continues but is less significant. The thermal effect could be due to rupture of low energy bonds in the collagen or collagen-ground substance aggregate. The method may be valuable for the study of changes of state of connective tissues in physiologic and pathologic conditions.

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