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The effect of temperatures above 37°C on collagen denaturation in a physiological environment

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Abstract:

Currently, no published study exists examining the effect of raised temperatures on Type 1 collagen fibril denaturation in a physiological environment. This study hypothesizes that some proportion of collagen in a physiological environment melts gradually as the temperature is increased above body temperature, and that the proportion will increase at a specific temperature with added time, until the rate of melting is slowed and a plateau is reached. This investigation will aid in a better understanding of how collagen reacts to any situation where the temperature of the connective tissue increases above normal. The temperatures measured were between 37°C (rat body temperature) and 45°C, at varying times from 0-2880 minutes. At specified times, aliquots of supernatant were drawn for analysis from a sample containing rat tail tendon collagen and complete supplement solution. Acid hydrolysis was performed and hydroxyproline assays were used to determine the amount of hydroxyproline in each sample. The hydroxyproline values were then converted into collagen amounts. In each case, aside from 44°C and 45°C, a plateau was reached where the percentage of collagen denatured did not significantly increase. At 37°C and 38°C, there was

little change of the percent denatured from start to finish. However, by 41°C, the melting occurring between 0 minutes and 240 minutes was significant. The only sample that completely melted was at 43°C. If given additional time, it appears that the 44°C and 45°C samples also would have fully denatured.

Introduction

Type 1 collagen, a strong and abundant triple helical protein found in vertebrate connective tissue, is the main extra-cellular constituent found in skin, bone, tendon, and dentin. Collagen is primarily composed of the amino acids glycine, proline, and hydroxyproline. This protein melts, or denatures, when the temperature is increased above normal. However, some regions of the molecule are less stable than others, therefore the molecule does not solely consist of all helix or all random coil when solid or denatured. Instead, melting occurs as a phase transition, with some parts of the molecules denaturing before others. Unlike most proteins, the denatured products are soluble (Piez, 1984).

Currently, no published study exists examining the effect of raised temperatures on collagen fibril denaturation in a physiological environment. In a previous study, it was determined that melting at a neutral pH can occur at 1-2C° (38°-39°C) above body temperature in several species, including collagen taken from rat tail tendon (Piez, 1984). However, it is not known if collagen behaves similarly when in the body. This study hypothesizes that some proportion of collagen in a physiological environment melts gradually as the temperature is increased above body temperature, and that the proportion will increase at a specific temperature with added time, until the rate of melting is slowed and a plateau is reached. This investigation of collagen fibril denaturation could aid in a better understanding of how it reacts to any situation where the temperature of the connective tissue increases above normal.

Materials and Methods

Dialysis tubing (Spectra/Por, 23mmx30m, MWCO 6-8000, vol/cm 1.7mL) was filled with purified Type 1 collagen from rat tail tendon (3.7mg/ml in 0.5M acetic acid + 0.05%

NaN_3, 3/22/99) and magnetically stirred in a .012N HCl + 0.05% NaN_3 solution overnight in a 4°C cooler. The solution was replaced each day for two days. When dialysis was complete, the collagen was stored at 4°C until ready for use.

For each temperature trial, three replicate microfuge tubes vortexed with 1 part collagen (now in .012 N HCl solution) and 0.4 parts of complete supplement solution (for final concentration of 5% Calf Serum, physiological for all components with a neutral pH) (Table 1) were placed in a circulating 37°C glycerin bath (simulating rat body temperature) to allow collagen fibril formation. After one hour, each microfuge tube was centrifuged at 16,000xG to pellet the solid state fibrils, had an aliquot of supernatant removed, and was transferred to a circulating water bath previously set at the appropriate temperature. Both glycerin and water bath temperatures were calibrated using the Thermo #6848 Ertco incubator thermometer, set by the US Department of Weights and Measures. The temperatures measured were between 37°C and 45°C, at varying times from 0 minutes to 2880 minutes (0-48 hours)(Table 2). At specified times, the microfuge tubes were centrifuged and aliquots of supernatant containing the denatured collagen were removed for analysis, with time outside of the desired temperature kept to a minimum. During the final aliquot time, both the supernatant and the pellet were individually placed in glass tubes for analysis. Aliquots were stored at -20°C in glass tubes until acid hydrolysis was performed. To each aliquot, specified amounts of HCl (6N HCl final concentration) were added and vortexed. Next, the tubes were placed in a 110°C oven for at least 18 hours to undergo acid hydrolysis to liberate the hydroxyproline. It has been previously demonstrated that these conditions will yield hydroxyproline and not have detrimental effects on it (Pashley et al., 1966). After hydrolysis, the samples were centrifuged to remove particulate matter, transferred to eppendorf tubes, and

stored at 4°C. All samples were then checked and adjusted to ensure that the appropriate volume of acid was present, and had not evaporated during hydrolysis. Hydroxyproline assays were then performed to determine the amount of hydroxyproline in a known standard series and in each sample (Bergman, 1961). Following the assay, the plate cooled for a maximum of 30 minutes and was read on a spectrophotometer at 555nm. The amount of hydroxyproline in each sample was determined from a hydroxyproline standard curve. The amount of collagen in each sample was determined by multiplying the hydroxyproline value by 6.7. Those replicates meeting the strict data acceptance requirements were then averaged to provide the final data. This study included a total of 200 samples.

Results

In general, the results show that the rate of melting increases for a certain period of time, and then plateaus (Fig 1). At 37°C and 38°C, there is little change between the percent of collagen denatured at 0 minutes through 2880 minutes (48 hours). At 39°C and 40°C, a small proportion of fibril denaturation occurred, and then a plateau was reached (22% and 26% respectively) where the percentage of collagen melting did not significantly increase. At 41°C, the melting occurring between 0 minutes and 240 minutes was very significant. By 240 minutes, 58% of the collagen was denatured. The amount then gradually increased until 2880 minutes, where 79% of the collagen had melted. The initial sample taken after only 120 minutes at 42°C showed an even more drastic change, as already 75% of the collagen was denatured. The only sample that completely melted was at 43°C after 300 minutes, although 94% of the melting took place within the first 60 minutes.

At 44°C and 45°C, the percentage of collagen melted increased so rapidly that it probably also would have melted completely, if additional time was allowed. Closer

inspection of the 44°C and 45°C samples reveal questionable results (Fig 2). The graph shows the percentage of collagen denatured to be increasing through 10 minutes, but then it plateaued.

Also, the initial percentage of denatured collagen at time 0 for 42°C, 43°C, and 44°C was higher than in the other samples. It is inconclusive why these initial values are higher, although the insoluble collagen fibrils may have been loosened between centrifugation and sampling, or an aliquot may have been drawn too close to the pellet, therefore removing some of the collagen fibrils.

Discussion

In general, the data prove the hypothesis to be correct, that some proportion of collagen melts gradually as the temperature is increased above body temperature, and that the proportion will increase at a specified temperature with added time, until the rate of melting is slowed and levels off. The results display a similar pattern to a previous study, although not performed in physiological conditions, that the rate of denaturation was slow for low temperatures, and the rate of denaturation increased for higher temperatures (Miles, 1993). It is also worthwhile to note that collagen in solution denatures at close to the maximum body temperature of the species of animal from which the molecules are extracted (Miles and Ghelashvili, 1999).

From 41°C to 43°C, there is an abrupt change in melting. Approximately before eight hours there is a rapid increase in the percentage being melted, and afterwards the percentage being melted stays somewhat constant. At 43°C, the plateau occurred after three hours, with nearly all of the collagen in solution (97%). Before 41°C, the percentage of collagen going

into solution does not change much. After 43°C, the collagen goes into solution even faster, although the experiment was not continued long enough to verify the plateau stages.

The large variation in the proportion of collagen denatured between the 44°C and 45°C samples could be attributed to the short time span of incubation at the specified temperatures. At these temperatures, portions of the collagen were in phase transition, not in pellet or in solution, but rather as a transparent viscous gelatin. Despite the effort, some portions of the collagen in phase transition could have been easily picked up while pipetting and thus skewing the results. Both 44°C and 45°C need further examination, with less interference during 0-10 minute incubation time, more uniform centrifugation times, and samples taken at longer periods of time, at least up to 60 minutes. In each case, aside from 44°C and 45°C, which were not sampled beyond 20 minutes, a plateau was reached where the percentage of collagen denatured did not significantly increase.

More study is needed to determine if the gradually increasing percentages during the plateau stage eventually could reach 100% melting in the physiological environment. Another area for additional research would be to learn what proportion of denatured collagen can recover from specific temperatures when returned to normal body temperature. According to Chen, a time-dependant partial recovery is possible when the sample is returned to normal body temperature (Chen et. al, 1997). Research topics such the one discussed in this paper and the topics for further discussion are all important in helping to gain a greater understanding of the role of collagen in the body when there is an increased temperature.

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Solution	ml	parts
0.24 N HCl	2	2
DME Salts A (10x)	2	2
DME Salts B (1000x)	0.02	0.02
Vitamin, etc. mix*	1	1
Calf Serum	1	1
NaN3	25mg	

Vitamin, etc. mix

Solution	parts
MEM Vitamin Solution (100X) (in 0.85% NaCl)	1
MEM Amino Acids (50X) without L-Glutamine	2
MEM Sodium Pyruvate Solution-100mM plus d-glucose 100mg/mL (100X)	1
L-Glutamine-200mM (100X) (29.2 mg/ml 0.85% NaCl)	1
Penicillin-Streptomycin (100X) (Penicillin G sodium (10,000 units/ml) and Streptomycin sulfate (10,000 ug or U/ml) all in 0.85% saline)	1

Table 1: Components of Complete Supplement Solution. This solution was added to the collagen solution to yield a final concentration of 5% Calf Serum and 1X Minimal Essential Medium (MEM).

37°C	38°C	39°C	40°C	41°C	42°C	43°C	44°C	45°C
0	0	0	0	0	0	0	0	0
240	240	240	240	240	120	60	2	2
480	480	480	480	480	240	180	4	4
720	720	720	720	720	360	300	6	6
1440	1440	1440	1440	1440	480	420	8	8
2880	2880	2880	2880	2880	720	540	10	10
					1440	720	20	20

Table 2: Chart showing the temperatures sampled and the time (min) at which supernatant was removed for analysis.

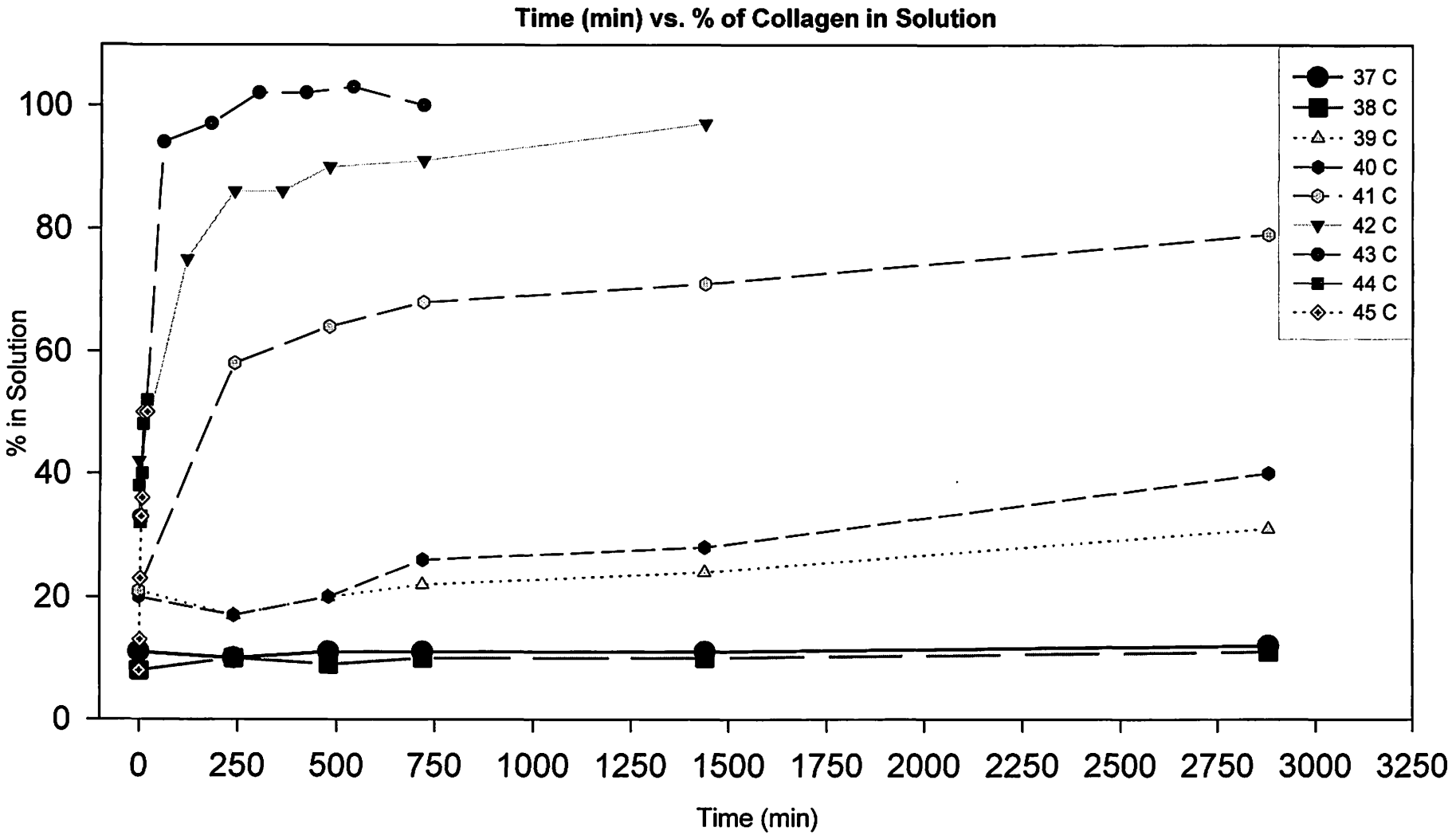

Figure 1: Time course of collagen denaturation at the specified temperatures over the range 37°C-45°C. For temperatures 37°C through 43°C, a plateau is reached, whereas 44°C and 45°C have not reached a plateau after 20 minutes. For each data point, N=3.

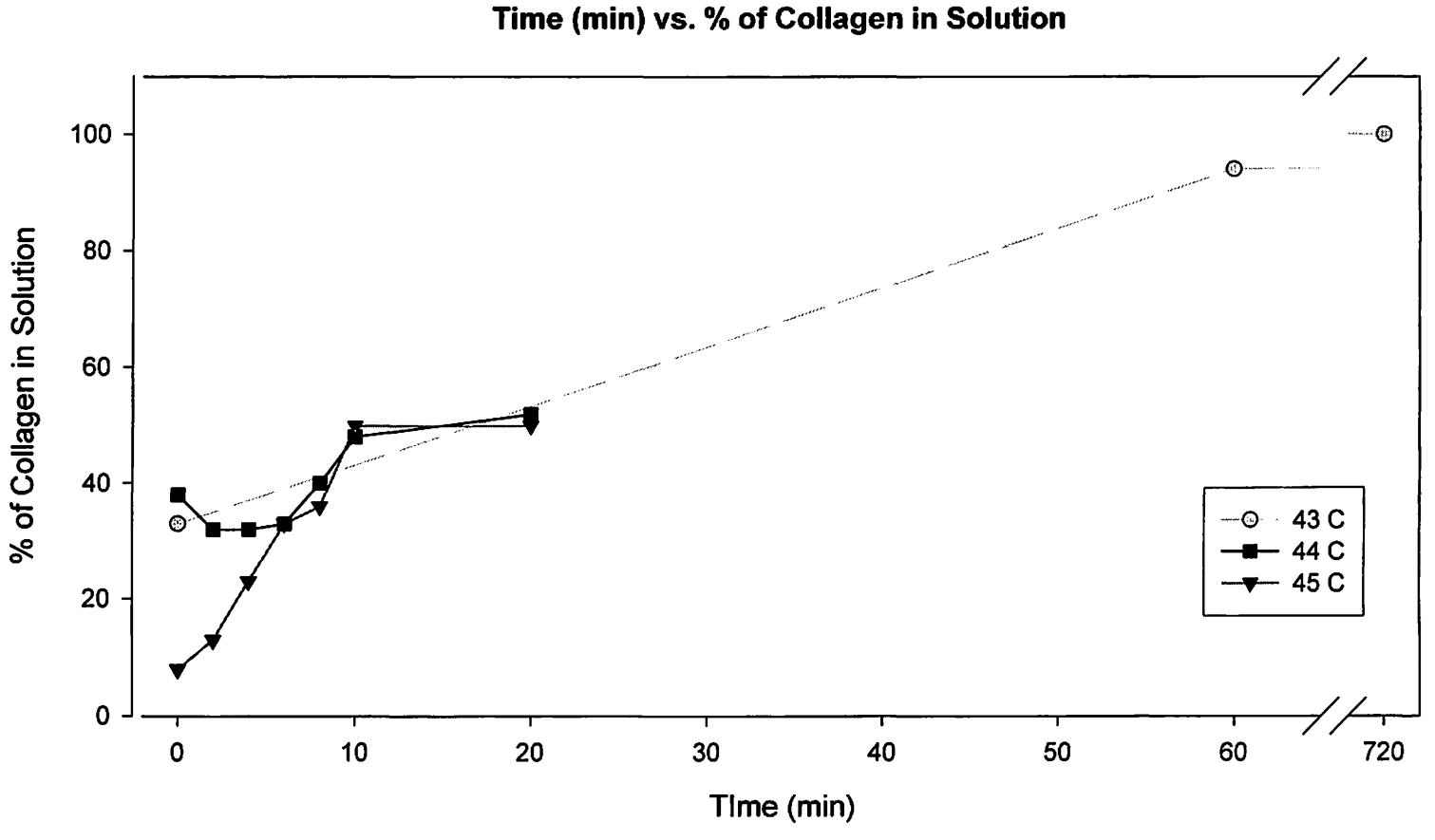

Figure 2: Enlarged graph displaying in closer detail the results of 43°C, 44°C, and 45°C. For each data point, N=3.