Preliminary Notes

PN 1254

Thermal transitions in collagen

It is well-known that when collagen is heated it undergoes a striking dimensional change quite abruptly when a characteristic shrinkage temperature, T_s, is reached. The shrinkage results directly from melting of the crystalline phase, but FLORY and his co-workers[1] have shown that the normal measurement of T_s may involve superheating by as much as 10° above the true melting point. By partially pre-melting their samples, FLORY AND GARRETT[2] obtained values of the equilibrium melting temperature, T_m, which varied with composition in agreement with polymer-melting theory.

It is not so widely appreciated that other structural changes take place in collagen at temperatures well below T_s or T_m. Thus FLORY AND GARRETT[2] have reported the existence of an apparent glass-transition temperature, T_g, between 40° and 45° in a beef Achilles tendon-water system with approx. 40% of collagen by weight. This transition, which was completely reversible, was determined dilatometrically and the sample was not subjected to any mechanical stress. Characteristic temperatures have also been found at which abrupt changes occur in the mechanical behaviour of collagen which is under stress. For example, RIGBY *et al.*[3] found that the rate of stress-relaxation of collagen fibres stretched by 1% in saline solution at different temperatures increased rapidly at approx. 40°; furthermore, a fibre could then no longer recover its original length when the stress was removed. The possibility thus arises that irreversible deformation in collagen (*i.e.* physiological damage) can result from the application of stress when the temperature is above the glass-transition point.

We now report some preliminary observations of transitions in collagen–saline systems particularly in the vicinity of 40°. Thermal expansion over the range 20–65° was measured for kangaroo-tail and beef tendons in 0.9% (w/w) aq. NaCl. By means of a buoyant-weighing technique, previously used for measuring glass-transitions in rubbers, specific volume–temperature curves were obtained giving clear-cut evidence of both glass-like and melting transitions. Following the procedure of FLORY AND GARRETT, the measurements were repeated after the specimens had been partially melted. Parallel with these experiments, force–temperature relations were obtained for fibres held at nominally constant length in an extensometer equipped with a force-transducer of very high stiffness. This is the preferred method for the determination of T_s, but it can also be used to detect other structural transitions as will be seen below.

Thermal-expansion curves for kangaroo tail tendon in saline are given in Fig. 1. Curve I, for the native tendon, shows a small, glass-like transition at approx. 40° and a first-order transition at 53°; the value of T_s obtained from the extensometer

Abbreviations: T_s, shrinkage temperature; T_m, equilibrium melting temperature; T_g, glass-transition temperature.

was 54°. After the partial melting produced in this test, the sample was cooled to room temperature and a repeat experiment on the next day led to Curve II with a glass transition at 38° and melting at 54°. In these tests the temperature at each point was held constant for 30 min or longer before taking the reading. Repeated experiments were also made on the beef tendon in which the temperature was increased at a constant rate (1° in 8 min) for better comparison with the extensometer values. The transitions were not so clearly marked as those in Fig. 1, but the specific volume of successive heating showed significant trends. T_g was reduced from 47.5 for the native tendon, successively, to 45°, to 43° and to 40° by one, two and three partial meltings, respectively. The first-order transition, however, was observed at 59° for all of these conditions. It should be emphasized that the sample was stored for approx. 18 h at 5° between tests.

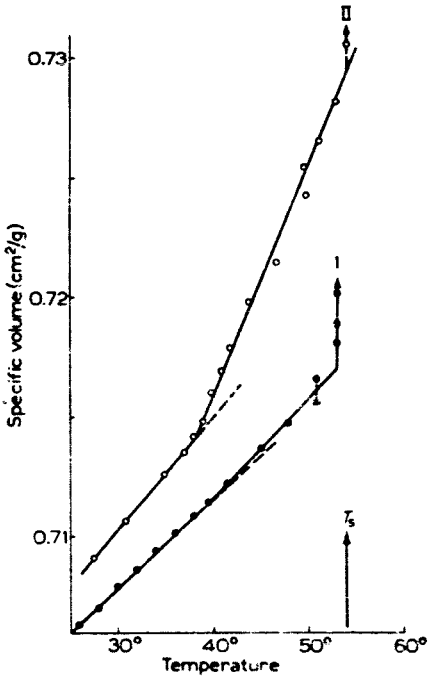

Fig. 1. Thermal expansion of kangaroo-tail tendon in 0.9% saline. ●—●, native material; ○—○, partially purified material.

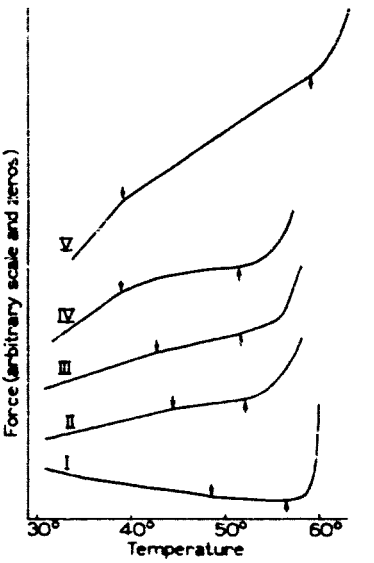

Fig. 2. Force-temperature relations for partly melted collagens. ↓, location of T_g; ↑, location of T_s.

A series of force–temperature curves for the beef tendon is shown in Fig. 2. Curve I is for the material in its native state; the force initially decreases with increasing temperature (the net result of decreasing modulus together with the differential expansion of specimen and clamping system), but shows a slight inflexion at about 48° and starts to increase rapidly at about 57°, the shrinkage temperature. Curves II, III and IV were obtained on the same specimen in succession during the course of a day, thereby meeting FLORY's condition of partial pre-melting to establish a fresh crystalline–amorphous interface. It can be seen how both transition points are progressively reduced, to approx. 39° and 51°, respectively. After completing

Curve IV the specimen was relaxed in the saline solution at room temperature for 48 h and then re-tested. The resulting Curve V shows that the shrinkage temperature had returned close to its original level, but that the lower transition point remained at 39°, the value produced by the successive heat treatments.

Comparison of these results indicates that both methods are in fact detecting the same two transition temperatures, *viz.* T_g and T_s. Thus for further structural investigations the relatively difficult determination of T_g by conventional methods can be replaced by the simple force–temperature technique.

The force–temperature results show that T_s was temporarily lowered by the pre-melting treatment but recovered its original value after standing at 20°. This is in agreement with FLORY's interpretation of the difference between T_s and T_m as a superheating phenomenon; after the establishment of a fresh crystalline–amorphous interface by partial melting, the value of T_s is reduced towards the equilibrium melting point T_m which in the present instance is apparently close to 51°.

We draw the following conclusions at this stage of the work:

1. A glass-transition occurs in native unstrained collagen under conditions resembling the state *in vivo*. For the mammalian collagens tested in the native conditions the temperature of this transition, T_g, lay between 40 and 50°.

2. T_g was lowered irreversibly to 40° or below by repeated heating of the sample to a temperature just above T_s. The magnitude of the transition increased with the proportion of collagen which had been melted.

3. T_g can be determined from a force–temperature experiment which is far simpler than the conventional methods.

The authors wish to express their gratitude to Mr. G. SHARMAN of C.S.I.R.O., Division of Wildlife Research and to Mr. P. BRYDON of the State Meat Inspection Service, N.S.W. Department of Agriculture, for the provision of the raw materials, as well as to Mr. G. GRAY for careful experimental assistance.

Division of Textile Physics, C.S.I.R.O., Wool Research Laboratories, Ryde, Sydney (Australia)

P. MASON
B. J. RIGBY

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Received March 4th, 1963

Biochim. Biophys. Acta, 66 (1963) 448–450

PN 1246

Nitrate reduction in the light by isolated chloroplasts

PANEQUE *et al.*[1] and LOSADA *et al.*[2] have shown that chloroplasts isolated from spinach leaves can reduce nitrite and hydroxylamine under suitable conditions. The mechanism involved in the reduction of these inorganic nitrogen derivatives was found to be similar to the one implicated in the photosynthetic reduction of TPN^+ (ref. 3). To take place, the reaction required in addition to light- or dark-reduced spinach ferredoxin, a thermolabile factor(s) also present in the chloroplast extract.

The photochemical reduction of nitrate has been investigated at the cellular level by WARBURG AND NEGELEIN[4], and VAN NIEL *et al.*[5] using suspensions of Chlorella cells. EVANS AND NASON[6] and JAGENDORF[7] have succeeded in coupling the photochemical reduction of TPN^+ by soybean and spinach grana to the reduction of nitrate by a purified nitrate reductase obtained from soybean leaves and Neurospora, respectively. More recently, however, HAGEMAN *et al.*[8] have [illegible] enzymic system from leaves of *Cucurbita pepo* and *Zea mays* which catalyzes the reduction of nitrate by reduced benzyl viologen, but not by TPNH in the absence of the dye. The present report is concerned with the light reduction of nitrate in a reconstituted chloroplast system. More details on the mechanism of this process will be presented in subsequent publications.

Washed broken chloroplasts and chloroplast extract were prepared from spinach according to WHATLEY *et al.*[9]. Spinach ferredoxin was obtained by the method of TAGAWA AND ARNON[3]. Ferredoxin-free chloroplast extract was prepared as described elsewhere[8]. Inactivation of the ferredoxin-free chloroplast extract was brought about by heating 5 min in a boiling-water bath.

The reactions were carried out under argon, at 20°, in Warburg vessels illuminated from below by a 100-W fluorescent lamp providing approx. 20000 lx. Nitrite and hydroxylamine were estimated by the method of NOVAK AND WILSON[10]. Ammonia was determined by nesslerization after distillation and absorption of the gas in 0.01 N H_2SO_4 in CONWAY units[11]. TPNH was estimated by measuring the absorbancy at 340 mμ.

TABLE I

PHOTOREDUCTION OF NITRATE BY CHLOROPLASTS

The reaction mixture included in a final volume of 3 ml: washed broken chloroplasts, heated at 55° for 5 min, containing 0.4 mg chlorophyll; ferredoxin-free chloroplast extract (Fd-free CE) equivalent to 1.5 mg chlorophyll; and the following in μmoles: potassium phosphate (pH 7.0), 200; sodium ascorbate, 20; 2,6-dichlorophenolindophenol, 0.2; KNO3, 60; benzyl viologen, 1. Reaction time, 45 min.

Addition	Nitrite formed (μmoles)
Complete	30
Benzyl viologen omitted	0
Fd-free CE omitted	0
Fd-free CE heated	18

Table I shows the light-dependent reduction of nitrate by a chloroplast system in which the photoevolution of oxygen has been suppressed, the electrons being supplied by the couple ascorbate–dichlorophenolindophenol instead of water[12,13]. The reaction did not proceed in the dark and required in addition to chloroplast fragments, benzyl viologen and a thermolabile factor (presumably nitrate reductase) present in the chloroplast extract. No change in activity was observed for nitrate concentrations varying from 2–20 mM. The product of nitrate reduction was found to be nitrite. Practically no hydroxylamine and ammonia were formed. In the absence of nitrate no formation of nitrite occurred when dialysed chloroplast extract was used but appreciable amounts of nitrite were produced if no dialysis was previously effected. Undoubtedly the chloroplast extract provided enough nitrate for the reaction to take place.

TABLE II

PHOTOREDUCTION OF TPN^+ BY CHLOROPLASTS IN THE PRESENCE OF NITRATE

The reaction mixture included in a final volume of 3 ml: washed chloroplast fragments containing 0.4 mg chlorophyll; spinach ferredoxin, 0.4 mg; and the following in μmoles: potassium phosphate (pH 7.0), 200; TPN^+, 5; KNO_3, as indicated. Reaction time, 12 min.

Nitrate added (μmoles)	*Oxygen evolved (μmoles)*	*$TPNH_2$ formed (μmoles)*
—	3.9	4.1
6	3.7	3.9
15	3.1	3.4
60	3.7	3.8

WHITELEY AND WOOLFOLK[14] have shown that methyl viologen is considerably more effective than *Micrococcus lactilyticus* ferredoxin in mediating the dark reduction of nitrate to ammonia by extracts of the same microorganism. Under our conditions (Table I), when benzyl viologen was replaced by either methyl viologen (1 μmole), spinach ferredoxin (0.4 mg), or spinach ferredoxin (0.4 mg) plus TPN^+ (0.3 μmole), no reduction of nitrate took place.

EVANS AND NASON[6] have reported, without giving experimental details, that the concentration of nitrate (about 20 mM) required for optimum nitrate reductase activity results in a striking decrease in the photochemical reduction of TPN^+, a reaction now known to be ferredoxin dependent[3]. We have found, however, that nitrate in concentration up to 20 mM does not inhibit the light-dependent reduction of TPN^+, with either water (Table II) or ascorbate as the electron donor. The possibility that nitrate could interfere with the electron-transport chain at the ferredoxin level must consequently be excluded.

This work was supported in part by a grant from the J. March Foundation.

Sección de Bioquímica y Fisiología Celular, Centro de Investigaciones Biológicas, C.S.I.C., Madrid (Spain)

F. F. DEL CAMPO
A. PANEQUE
J. M. RAMIREZ
M. LOSADA

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Received February 28th, 1963