SHRINKAGE OF COLLAGEN

by

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X-ray diffraction patterns have shown that heat shrinkage of native collagen involves conversion from a predominantly crystalline state to an amorphous state (ASTBURY[1]). It has been suggested that this conversion ruptures the links between polypeptide chains, and the chains are then able to fold and assume a more stable configuration (MEYER[21] and BRAYBROOKS, MCCANDLISH, AND ATKIN[4]). Thus re-examination and extension of studies on collagen shrinkage should promote understanding of structural changes in proteins of the type involved in denaturation. Moreover, such studies have a direct bearing on certain aspects of some important industries.

METHODS

Preparation of tissues

Sheepskin. The skin was placed in a refrigerator at 4° within 1 h of flaying and sampled as required. The wool was shorn close to the surface, and strips, measuring 0.5 cm ± 0.1 cm by approximately 5 cm, were cut for testing. Samples of the same dimensions were cut for testing the collagen preparations. The mean skin thickness was 0.2 cm and the cross-sectional area was therefore 0.1 cm^2.

"Collagen" preparations from sheepskin. Preliminary tests showed that digestion of certain components of the skin by partial or complete sweating at 25°, or by incubation for 17 h at 25° in 0.4% trypsin (Difco 1:250), 0.4% papain (Parke Davis), or in an aqueous extract containing a mould protease did not affect the shrinkage temperature (s.t.) when heated in water or in the enzyme extracts. However, enzyme digestion alone is insufficient to remove all non-collagenous constituents and other methods were therefore applied. Liming, followed by neutralization with acetic acid, a treatment normally recommended in the preparation of collagen from hide (HIGHBERGER[12]) or from goat skin (THEIS AND JACOBY[24]) lowered the s.t. Thus immersion of skin in saturated $Ca(OH)_2$ for 17 h at 20°, deliming in dilute acetic acid at p_H 4.0 for 2 h and washing in running water for 2 h reduced the s.t. from 67° to 61°.

In view of these findings a drastic procedure was adopted which, though certain to lower the s.t., should remove more of the non-collagenous components than other methods. A pickled pelt from a skin fellmongered by the painting process was used, and the thermostat layer, containing the sebaceous and suderiferous glands, and the fatty layer were removed from the reticular layer of the dermis in a tannery shaving machine. It was washed in running water for 20 h, neutralized in 0.4% $NaHCO_3$ for 4 h, washed in running water for 6 h, digested for 18 h in 0.1% trypsin (Difco 1:250) containing

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0.5% $CaCO_3$ and 0.5% toluene, washed in water for 3 h, dehydrated and degreased twice with ethanol for 2 h, then twice with acetone for 2 h, air-dried, digested with trypsin and dried with ethanol and acetone as before, soaked in benzol for 3 days to remove adhering fat, rinsed twice in acetone, and finally dried in the air. This preparation, termed "Collagen A", had a s.t. of 55°. Removal of traces of Ca by extraction three times in 24 h with 1.0 M CH_3COOH and neutralization by repeatedly adjusting the wash water to p_H 7 yielded "Collagen AI" having a s.t. of 53°.

Rat tail tendon. Tendons were dissected from adult rats tails and held at 4° in water under toluene for periods ranging up to 4 days without change in the s.t. Fibres, approximately 0.04 cm in diameter, that is 0.00126 cm^2 in cross-sectional area, and 3 cm long, were pulled from the tendons for testing.

Measurement of shrinkage temperature

Thread was attached to the ends of each strip of skin or collagen, leaving 4 cm between the points of attachment, one end was attached to a wire projecting from the base of a 250 ml beaker containing 200 ml of the liquid to be tested and the other to an aluminium lever which recorded contraction of the strip on the smoked drum of a kymograph. The lever applied a load of 5 g weight to the strip causing slight stretching. After completion of the soaking period the liquid covering the specimen was agitated continuously by a mechanical stirrer and heated to raise its temperature at the rate of 10° per min, and the recording drum was rotated at 1 cm per min. The temperature was marked on the curves at each successive 5° increase and at the s.t. The tendon fibres were tested similarly except that the cotton threads were attached to the fibres at points 2.5 cm apart. Both Collagen A and tendon fibres ruptured when heated in water beyond the s.t.

To test organic compounds available only in small quantities, a micro method was employed which required only 1 ml of solution. A small piece of skin or tendon was attached to the bulb of a thermometer and immersed in the solution to be tested in a test tube, 1 cm internal diameter. The tube was heated in a water bath at the same rate as in the macro method and the temperature noted at which shrinkage became apparent. The results for salicylic acid (p_H 7.0) in Fig. 1, show that the s.t. at zero load was lower than the s.t. at 50 g weight per cm^2 and the difference increased with concentration. Similar results were obtained using NaCNS solutions. The three values obtained by the micro method were therefore corrected to the corresponding approximate s.t.'s. at 50 g weight per cm^2 by adding the appropriate differences from Fig. 1. Some compounds, such as *p*-

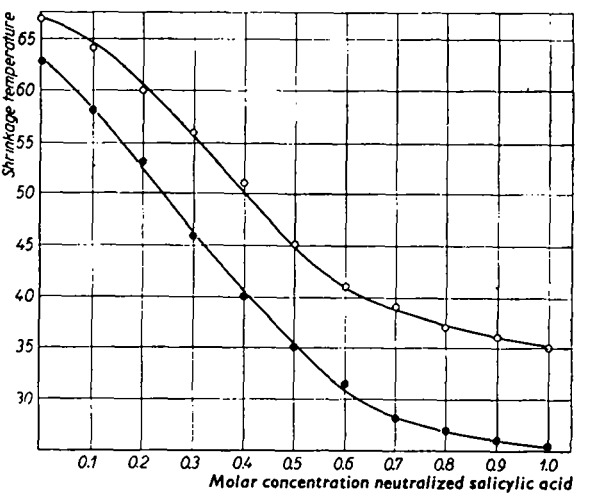

Fig. 1. Effect of concentration of salicylic acid (p_H 7) on the s.t. of sheepskin
o at a load of 50 g weight per cm^2-measured by the standard method.
• at zero load-measured by the micro method.

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phenylene-diamine and sodium dodecyl sulphate, which were incompletely soluble at room temperature, dissolved on warming at a temperature below the s.t.

Preparation of solutions for testing

Unless otherwise stated, all the compounds examined were tested at 1.0 M concentration and the solutions were adjusted to p_H 7.0 with HCl or KOH before testing. The glass electrode was used in making all p_H adjustments and measurements. p_H 7.0 is probably close to the isoelectric point of unlimed collagen (HIGHBERGER[13]; BEEK AND SOOKNE[2]). When the p_H shifted towards neutrality during the test, as it did in unbuffered solutions in the p_H range 4 to 10, the values obtained subsequent to testing are reported since they represent more nearly the p_H of the solution in equilibrium with the tissues during shrinkage.

Time of soaking

A standard soaking period of 1.5 h before measuring the s.t. was adopted, since this was found to be sufficient for the establishment of a constant s.t. In the highly concentrated solutions used in the elasticity experiments 10 min soaking was sufficient to shrink the tissues.

Elasticity of tissues

The elasticity of shorn skin and collagen A was measured by cutting strips of these materials of the width and thickness used for s.t. measurement but 7 cm long, attaching threads 0.5 cm from either end, tying one to a fixed wire at the base of a beaker and the other to the hook of a calibrated 500 g spring balance, and applying load by lifting the balance vertically by winding an attached cord on a drum. The length of the strip between the two points of attachment was measured to 0.1 cm for successive loads up to the elastic limit. At zero load the skin was slightly curled and its length could not be accurately measured. Extrapolation of the load-extension curve for several experiments in which the skin was immersed in water showed the length at zero load to be 92.5% of the length at 5 g load. Since these results were reproducible the initial length of each strip could be calculated from the length at 5 g load, thereby obviating the necessity for determining the load-extension curve for each sample.

The elasticity of 0.04 cm diameter tendon fibres was measured similarly except that the length of tendon between the points of attachment was 2.6 cm.

RESULTS OF SOME PRELIMINARY EXPERIMENTS

Influence of direction on s.t. of skin

Strips cut from a piece of sheepskin 10 cm square at angles of 0°, 45°, 90° and 135° to one edge all shrank at 67° temperature within $\pm$ 0.5°. The orientation of the strip in the skin is therefore unimportant.

Thermostat and reticular layers of skin

Dissection of strips of sheepskin along the fatty layer yielded samples of the thermostat and reticular layers poor and rich, respectively, in collagen bundles. Although both shrank at 67°, the extent of shrinkage of the reticular layer was approximately three times that of the thermostat layer. During shrinkage therefore, whole skin curls with the reticular layer innermost.

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Effect of tension applied during the shrinkage

The influence of tension on the s.t. of skin and tendon was measured by applying known loads by the method employed for measuring the elasticity of tissues, but using a more sensitive spring balance calibrated up to a load weight of 12 g. The s.t. was judged by noting the temperature at which the specimen began to contract, movement being detected by sighting it against a millimeter scale. Fig. 2 shows that the s.t. was elevated several degrees by increasing the load from zero to 5 g but little beyond that weight. The curve for tendon fibres continues to rise steeply at loads in excess of 3 g weight. It should be noted that the mean cross-sectional area was only 0.00126 cm^2, whereas that of the skin was 0.1 cm^2. Owing to the difficulty of detecting the onset of shrinkage visually, the apparent values under 5 g load are higher than the correct values obtained for the same load from kymograph records. Although the s.t. at zero load for skin, shown in Fig. 2, was 64.5°, most samples of skin tested shrank at 63°.

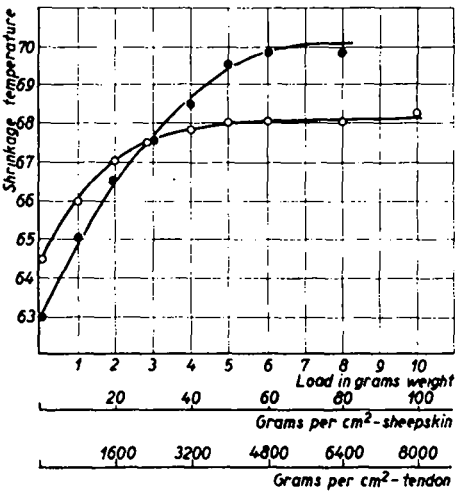

Fig. 2. Effect of load on shrinkage temperature in water
o sheepskin • tendon

Effect of moisture content

Skin was air-dried in one experiment for 3 days, and in another by holding *in vacuo* over H_2SO_4 for 3 days. Samples were taken at intervals during immersion in water for estimation of moisture content by drying to constant weight at 105° and for measurement of s.t. (Table I). The true moisture contents at the instant of shrinkage would be slightly higher than those reported, particularly after short periods, but this does not obscure the fall in s.t. with increase in moisture content.

TABLE I

RELATION BETWEEN TIME OF SOAKING DRY SKIN, MOISTURE CONTENT AND SHRINKAGE TEMPERATURE (s.t.)

Soaking period (h)	Air-dried skin		Vacuum-dried skin	
	Moisture content (%)	s.t.	Moisture content (%)	s.t.
Nil	11.2	90	12.6	90
0.5	—	73	—	80
1.0	46.2	70	43.4	73
2.3	54.6	67	60.6	70
24.0	72.0	68	74.2	67

A similar fall in the s.t. of collagen A during soaking is also evident from the following figures:

Time of soaking, in h:	nil,	0.5,	1.0,	6.5
S.t.:	65	60	57	55

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In spite of this effect no difference was detected between the moisture contents of unshrunk and shrunk samples of collagen A in vapour phase equilibrium with one another.

Elevation of s.t. of tendon fibres by withdrawal of moisture was also demonstrated by heating them in concentrated sucrose solutions. In 1.0 M sucrose the s.t. was elevated to 70°, and in 2.0 M solution to 72°. Similarly in 8 M ethanol the s.t. of tendon was elevated to 70° and in 12 M ethanol to 81°.

Varying the concentration of the shrinkage reagent

Progressive lowering of s.t. with increase in concentration of several shrinkage reagents is shown in Fig. 3. The curve for KCNS resembles that reported by KÜNTZEL[20]

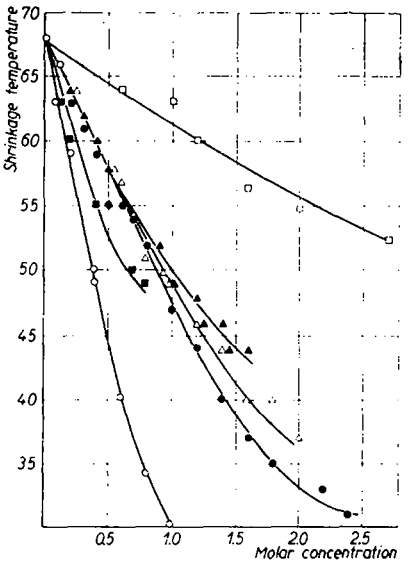

Fig. 3. Effect of concentration on shrinkage temperature, at p_H 7

• KCNS △ $NaClO_4$ ▲ KI
○ salicylic acid, ■ guanidine, □ urea

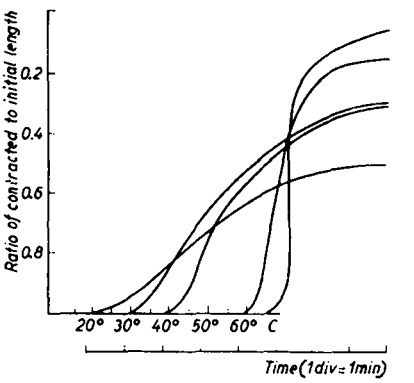

Fig. 4. Reduction in amount of skin shrinkage with increase in concentration of salicylic acid. Reading from left to right along the abscissa, the curves are for the following concentrations of salicylic acid (adjusted to p_H 7.0): 1.6 M, 1.0 M, 0.8 M, 0.2 M, nil.

Reduction in the rate and extent of shrinkage with increase in the concentration of salicylic acid (neutralized to p_H 7.0) used for soaking at room temperature is evident from Fig. 4, in which several shrinkage curves are superimposed. Partial shrinkage had occurred at room temperature, the amount increasing with the concentration of salicylic acid. Shrinkage was completed by heating in the same solutions.

Shrinkage of skin after soaking in salicylic acid and washing in water

In Fig. 5 it is shown that when skin was soaked in 1.0 M salicylate for 20 h at 32° and well washed in running water, the s.t. lowering influence of the salicylate was almost entirely removed, but if appreciably shrunk by heating in the salicylate to 37° before washing, shrinkage recommenced at a temperature intermediate between 40°, the value expected if none of the reagent was removed, and 67°, the value expected if removal was complete. Comparison of the salicylate curve with that for skin, partially shrunk in water before washing and reheating, shows that elevation of the temperature at which

shrinkage recommenced was not attributable merely to the change in physical state of the skin, but was apparently due to the use of salicylate to produce this change. Persistent effects of soaking in salicylic acid could be due to residual chemical or to a change in the structure or condition of the collagen.

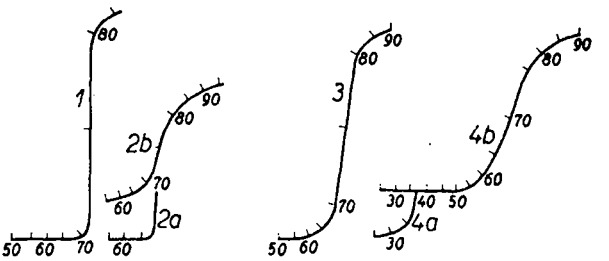

Fig. 5. Resumption of heat shrinkage after partial shrinkage and cooling

Curve 1. Heated in water to 62°, washed for 20 h in water and heated again. (Curve represents contraction during second heating).
Curve 2A. Heated in water to 70°
Curve 2B. Following treatment 2A, sample washed for 20 h in running water and heated in water
Curve 3. Heated in 1.0 M salicylic acid (pH 7.0) to 37°, washed for 20 h in water and heated again. (Curve represents contraction during second heating).
Curve 4A. Heated in 1.0 M salicylic acid (pH 7.0) at 37°
Curve 4B. Following treatment 4A, washed for 20 h in running water and heated in water

Elasticity before and after shrinkage in hot water or in thiocyanate

As shown in Fig. 6, sheepskin extended to 1.23 times its initial length at a load of 4500 g per cm² and contracted to 1.15 times on removing the load. Skin shrunk to 0.42 times its original length by heating in water for 10 min at 70° and cooling showed extension similar to that of unshrunk skin under the same load. However, while under the influence of either heat or concentrated NaCNS the skin displayed long-range elasticity, extending in the NaCNS to more than four times its shrunk length. Skin which was stretched in water at 70° ruptured when the load reached 3000 g per cm².

Similar results were obtained in another experiment in which various strips, some shrunk in hot water and others in CNS^-, were removed from these liquids before testing the elasticity. Washing one of these strips for 20 h in running water, following complete shrinkage in 10 M KCNS, caused elongation of the strip to about half its original length. Some KCNS may have remained in the tissues, for on heating in water contraction

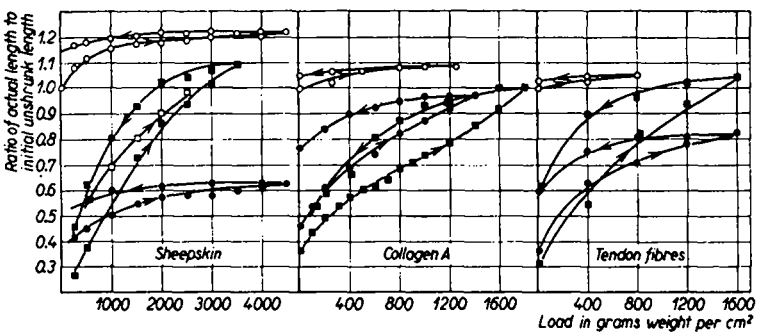

Fig. 6. Elasticity curves for sheepskin, collagen A and tendon fibres

↗ Increasing load ↙ Decreasing load

○ Unshrunk and tested at 18°
□ Shrunk and tested in water at 70°
• Shrunk in water at 70° and tested in water at 18°
■ Shrunk and tested in 10 M NaCNS at 18°

occurred at 40°, but shrinkage was only half of that strip which was not shrunk in KCNS but which had been washed in water for the same period. By heating a strip to 70° in

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water and cooling to room temperature while holding it at its original length, it was set in the extended state.

The curves for collagen A and tendon fibres in Fig. 6 show that, like sheepskin, both tissues displayed restricted elasticity before shrinkage and long-range elasticity in NaCNS. However, they differed from sheepskin in showing greater elasticity when cooled after shrinking in water at 70°. The extension of collagen A after heating and cooling almost equalled its extension in NaCNS solution.

THE EFFECTS OF INORGANIC IONS AND p_H

Salts containing ions of the lyotropic series

The s.t. values for the Na and K salts of a variety of inorganic acids tested at p_H 7 and 1.0 M concentration are shown in Table II. The collagen shrinkage activity of these anions agrees with their generally accepted position in the lyotropic series and with their increasing order of hydration.

TABLE II

S.t.'s OF SAMPLES IMMERSED IN 1.0 M SOLUTIONS OF SALTS CONTAINING ANIONS OF THE LYOTROPIC SERIES, AT p_H 7.0. S.t. IN WATER: SKIN 67°, TENDON 68°

	K salt		Na salt	
	Skin	Tendon	Skin	Tendon
CNS^-	47	50	50	52
I^-	50	50	49	50
ClO_4^-	--**	--**	49	51
NO_3^-	58	65	59	65
Br^-	60	66	59	62
NO_2^-	64	64	63	67
Cl^-	66	70	65	65
$Fe(CN)_6^{---}$	70	75	—	—
$H_2PO_2^-$	72	72	71	75
F^-	77	80	72	78
HPO_4^{--}*	78	81	77	85
SO_4^{--}	(79)**	(76)**	77	83

* At p_H 7 the phosphate would form a mixture: HPO_4^{--} and $H_2PO_4^-$

** Owing to low solubility, $KClO_4$ was not tested and K_2SO_4 was tested at 0.4 M concentration.

Of the cations tested (Table III), the alkaline earths were all equally effective and, as a class, they exhibited greater activity than the alkali metals. When tested at 0.5 M concentration, that is, at a concentration equivalent to that of the alkali metals, the alkaline earths were again the more effective. Each gave a s.t. of 55°. With the univalent alkali metals however, the shrinkage increased with increase of hydration.

It will be observed that in both the anion and cation series some of the ions raised the s.t. while others lowered it.

Molal solutions of certain oxidizing agents, $KClO_3$, $KBrO_3$ and KIO_3 gave s.t.'s with skin at 64°, 64° and 66°, respectively. Some heavy metal protein precipitants gave the following s.t.'s at the p_H values indicated: $CuSO_4$ (p_H 4.0) 58°, $FeCl_3$ (p_H 1.5) 60°, $AgNO_3$ (p_H 7.0) 55°, $Pb(CH_3COO)_2$ (p_H 7.0) 50°. Some compounds containing N in the anion gave the following values for skin and tendon respectively: sodium nitroprusside $Na_2Fe(CN)_5NO$ 40° and 32°, sodium azide NaN_3 58° and 57°, KCNO 67° and 68°.

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TABLE III

S.t.'s OF SAMPLES IMMERSED IN 1.0 M SOLUTIONS OF SALTS CONTAINING CATIONS OF THE LYOTROPIC SERIES, AT p_H 7.0. S.t. IN WATER: SKIN 67°, TENDON 68°

Cation	Chloride		Sulphate		Relative hydration (WASHBURN AND MILLARD[36])
	Skin	Tendon	Skin	Tendon	
Ca^{++}	50	50	—	—	—
Sr^{++}	50	50	—	—	—
Ba^{++}	50	50	—	—	—
Mg^{++}	52	56	66	70	—
Li^{+}	59	61	70	78	14
NH_4^{+}	63	63	73	78	—
Na^{+}	66	65	77	83	8.4
K^{+}	69	70	(79)*	(76)*	5.4

* See second footnote to Table II.

Rupture* of tendon fibres sometimes occurred in salt solutions at p_H 7.0 without prior shrinkage, but in a series of experiments the temperature of rupture was close to the s.t. (usually a degree or two higher), and in some instances it is therefore reported in the tables instead of the s.t. Owing to greater variability in both the s.t. and rupture temperature of tendon fibres less reliance should be placed on these values than on the corresponding values for collagen or skin.

Effect of p_H

In the absence of salts the s.t. of sheepskin remained constant within the range p_H 4 to 11, but fell sharply outside these limits (Fig. 7). At p_H 13.4 the damage was such that, after contraction, the strip commenced to stretch prior to rupture and the shrinkage curve resembled those for collagen A and tendon. From Fig. 7 it will be noted that not only was the s.t. of the collagen preparations considerably lower than the s.t. of skin but the range of stability of collagen AI to p_H was narrower.

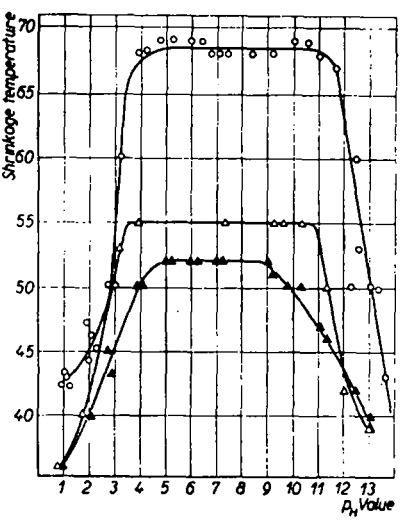

Fig. 7. Effect of p_H value on shrinkage temperature of sheepskin and skin collagen preparations
○ sheepskin △ collagen A
▲ collagen AI

An experiment was carried out which showed that the use of acid for deliming was primarily responsible for the low s.t. of the collagen preparations used in the present studies, and likewise of those employed by previous workers. Strips of fresh skin were soaked for 19 h in water adjusted to various p_H values between 1 and 13, the p_H being readjusted to the initial value after 2 h and 4 h. After washing in water, samples which had been soaked in acid solutions were neutralized in $KHCO_3$ solutions at p_H 9, and those soaked in alkaline solutions were neutralized in acetate buffer at p_H 5. After 27 h in these solutions, indicators applied to the freshly cut edges showed that the p_H values

* When used without qualification in this paper the term "rupture" signifies "rupture of tendon fibre without prior shrinkage".

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of all the strips lay within the p_H range 6 to 8. They were finally washed for 4 h in running water to remove salts. The s.t.'s of these strips, listed in Table IV, show clearly that at 20°, immersion in acid solution at or below p_H 4.0 weakened skin collagen, but alkali, even at p_H 12.8, was without effect and saturated $Ca(OH)_2$ elevated it slightly. When heated in solutions of similar alkalinity, however, collagen is severely damaged.

TABLE IV

EFFECT OF 19 h SOAKING AT 20° IN SOLUTIONS OF VARIOUS p_H VALUES ON S.t. OF NEUTRALIZED TISSUES

Reagent	Final p_H value	S.t.	
		Sample A	Sample B
HCl	1.3	52	53
,,	2.7	57	56
,,	4.0	62	61
KOH	7.2	66	66
,,	9.4	66	67
,,	10.7	66	66
,,	12.8	68	66
$Ca(OH)_2$	12.8	70	70

Varying the p_H of salt solutions

Fig. 8 shows that 0.5 M solutions of Na_2SO_4, KCl, KI, $NaClO_4$ and KCN exerted a constant effect on the s.t. at p_H values between 4 and 11. Their curves are therefore parallel in this range and maintain their relative positions up to p_H 14. In general, however, the relative positions of the curves for all the salts were reversed below p_H 2.5.

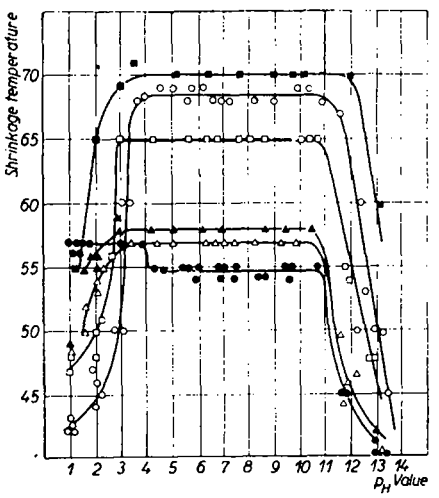

Fig. 8. p_H curves for sheepskin in the presence of 0.5 M solutions of salts containing various anions

○ No salt • KCNS △ $NaClO_4$ ▲ KI □ KCl ■ Na_2SO_4

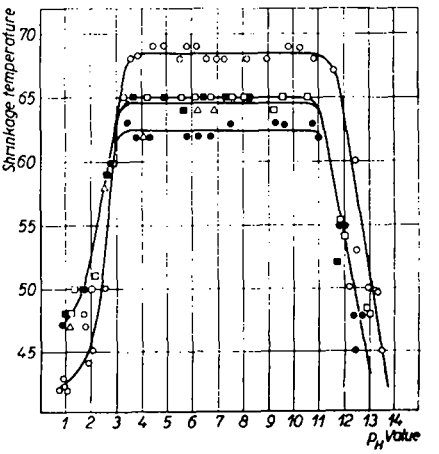

Fig. 9. p_H curves for sheepskin in the presence of 0.5 M solutions of salts containing various cations

○ No salt • LiCl △ NH_4Cl ■ NaCl □ KCl

The cations all lowered the s.t. above p_H 3.5 and displayed no reversal of lowering below p_H 2.5 (Fig. 9). Owing to serious loss of NH_3 on heating, NH_4Cl was not tested above p_H 7.

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TABLE V

S.t.'s OF SKIN AND TENDON, TESTED IN THE SAME SOLUTIONS

Salt at 0.5 M concn	Skin		Tendon		Collagen Al	
	pH 1.0	pH 2.0	pH 1.0	pH 2.0	pH 1.0	pH 2.0
Nil	42	46	38	30	37	42
KCl	47	48	54	52	37	37
KI	49	55	55	56	40	41
$NaClO_4$	48	53	53	54	40	40
KCNS	55	55	55	55	40	40

The s.t.'s for tendon were more variable than those for skin, and it was therefore impossible to represent the p_H-s.t. relationships by smooth curves. From the general trend of the results it was evident that the effect of p_H and the effect of anions and cations on the s.t. resembled those observed for skin. Reversal of the order of s.t. lowering below p_H 2.5 was not observed, however, and this is confirmed by the results presented in Table V. These values were obtained on the same day with the same solutions under identical conditions. Many of the tendons tested between p_H 1 and p_H 7 both in the presence and absence of salts ruptured without prior shrinkage. By plotting the percentage ruptured against the various p_H ranges, histograms showing the distribution were obtained (Fig. 10). The mean of the p_H values, at which rupture occurred in the s.t. experiments to compare various anions, is 4.08 and the standard deviation 1.33 p_H units. The cation figures have a mean of p_H 4.49 and standard deviation of 1.52 p_H units. Examination of the individual shrinkage curves for tendon showed that the extent of shrinkage increased steadily from almost nil at about p_H 6 to a maximum at p_H 9.5. There was also a tendency for the s.t. to rise to a maximum at about this value.

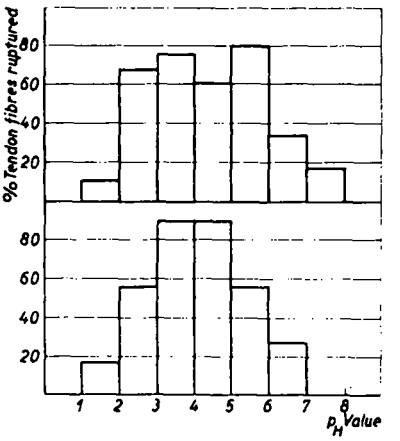

Fig. 10. Histograms showing percentage of tendon fibres ruptured in various p_H ranges during determination of s.t. Lower curve corresponds to data for anions. Upper curve corresponds to data for cations.

In the absence of salts the percentage of tendons which ruptured in acid solution was much higher. The following figures were obtained by testing 10 fibres at each p_H value:

p_H	1.0	2.0	3.0	3.9	4.6	5.7	6.7
Percentage ruptured before heating	nil	80	100	80	30	10	nil
Percentage ruptured before or during heating	20	100	100	100	90	30	10

The mean deduced from values for rupture before or during heating is p_H 3.5, and the standard deviation 1.29 p_H units.

The influence of p_H on the swelling of tendon fibres was studied by determining the ratio of their diameter after immersion in water adjusted to various p_H values to the initial diameter. The mean of five measurements of diameter along each fibre was obtained with the aid of a microscope and an eyepiece micrometer. The ratios, reported

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in Fig. 11, reached a maximum at p_H 3.0 in HCl solutions and at p_H 3.6 in oxalic acid solutions. The p_H values were checked before and after use. Attempts were made also to determine the p_H of maximum swelling in acetic acid, but the high concentration of acid required produced complete gelatinization at p_H 2.0 and p_H 2.5.

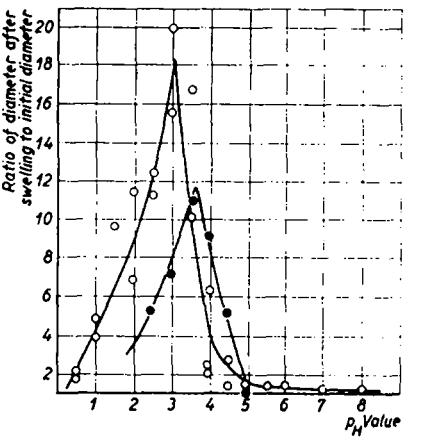

Fig. 11. Swelling of tendon fibres after 15 min at the p_H values indicated
○ p_H adjusted with HCl
• p_H adjusted with oxalic acid

THE EFFECT OF ORGANIC IONS

Fatty acids and aliphatic primary amines

After an initial rise in the s.t. of skin and tendon fibres from 67° to 72° with 1.0 M formic acid and to 74° with 1.0 M acetic acid, a mean reduction of about 5.5° was observed per C atom increment in the length of the chain in the following series of normal fatty acids: acetic acid, propionic acid, butyric acid, valeric acid, and caproic acid, that is up to the C_6 member of the series. Some additional reduction in s.t. probably occurred with lengthening of the chain up to the C_9 member of the series but, owing to the variability of the results, the position of the curve is uncertain (Fig. 12).

A steady fall in s.t. with increase in length of the C chain was also observed in the following series of normal aliphatic primary amines; methylamine, ethylamine, propylamine, butylamine and amylamine (Fig. 12). Unlike the first and second members of the fatty acid series, all the amines reduced the s.t. Moreover, increase in the length of the C chain beyond the C_5 member produced no further appreciable reduction in the s.t., and the mean reduction in s.t., approximately 3.8° per C atom increment in the length of chain, was less than that observed for the fatty acids. It will also be noted that individual amines produced lower s.t. 's than fatty acids having the same length of C chain.

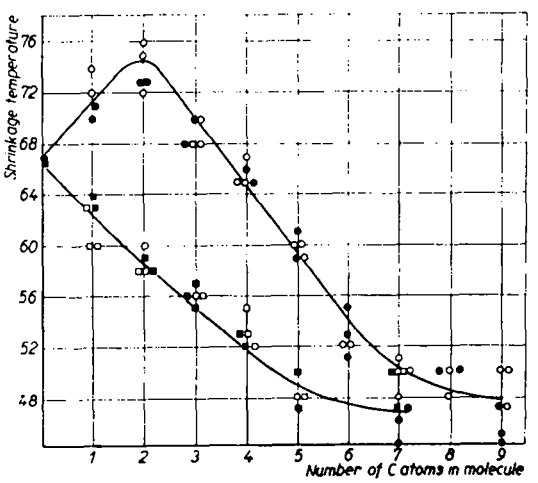

Fig. 12. Progressive lowering of s.t. with increase in length of carbon chain in the fatty acids and aliphatic primary amines, tested at 1.0 M concentration and neutralized to p_H 7
○ fatty acids acting on sheepskin
• fatty acids acting on tendon fibres
□ aliphatic primary amines acting on sheepskin
■ aliphatic primary amines acting on tendon fibres

Miscellaneous aliphatic acids, bases and un-ionized compounds

One of the most striking observations was that all the aliphatic dicarboxylic acids examined, and also citric acid, elevated the s.t. (Table VI). The elevation was more pronounced with tendon fibres than with skin. Among the amines it should be noted that s.t.'s produced by diethylamine and

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dipropylamine were close to those produced by the corresponding primary amines, ethylamine and propylamine. The water-soluble un-ionizable organic compounds examined exerted little, if any, effect on the s.t. at 1.0 M concentration. HCHO, which elevated the s.t. very considerably, is a widely used tanning agent.

TABLE VI

EFFECT OF 1.0 M SOLUTIONS OF SOME ALIPHATIC ACIDS, BASES AND UNIONIZED COMPOUNDS, NEUTRALIZED TO pH 7, ON THE S.t. OF COLLAGEN. S.t. IN WATER: SKIN 67°, TENDON 68°

Compounds	S.t.	
	Sheepskin	Tendon
Dicarboxylic Acids		
Maleic acid	82	82
Fumaric acid	80	79
Sebacic acid (pH 8.4)	73	75
Adipic acid	78	76
Succinic acid	76	82
Malonic acid	78	83
Oxalic acid	78	78
Tartaric acid	79	85
Miscellaneous Acids		
Trichloracetic acid	38	36
Hydrogen dodecyl sulphate*m	47	43
Iodoacetic acid	53	51
Bromobutyric acid	58	61
Methylamyl sulphosuccinic acid*	59	58
Bromoacetic acid	64	64
Ascorbic acid	64	71
Bromopropionic acid	67	63
Chloroacetic acid	67	67
Gluconic acid	68	70
Glycollic acid	68	71
Thioglycollic acid	69	70
Lactic acid	71	68
Citric acid	79	80
Secondary Amines		
Dipropylamine	55	55
Diethylamine	60	60
Miscellaneous Nitrogen Compounds		
Guanidine	48	43
Allylamine	57	54
Formamide	57	60
Triethanolamine (pH 8)	58	54
Acetamidine	59	59
Ethylene diamine	59	62
Thiourea	60	57
Malonamide	61	58
Urea	64	65
Acetamide	65	60
Glycine	68	74

* Pure sodium salts of these detergents were used.
m Tested by the micro method and corrected.

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TABLE VI (continued)

Compound	S.t.	
	Sheepskin	Tendon
Non-ionized Compounds		
2:3 Butane diol	63	63
Dioxan	65	65
Butyl alcohol	65	67
Acetone	66	70
Sucrose	67	67
Isopropyl alcohol	70	67
Ethyl alcohol	67	68
Methyl alcohol	70	70
Formaldehyde	90	85

TABLE VII

EFFECT OF 1.0 M SOLUTIONS OF SOME AROMATIC AND HETEROCYCLIC COMPOUNDS AT p_H 7. S.t. IN WATER: SKIN 67°, TENDON 68°

Compound	S.t.	
	Sheepskin	Tendon
Phenylpropiolic acid	30	20*
o-Hydroxy benzoic acid (salicylic acid)	33	
Cinnamic acid (p_H 8.6)	35	20*
Gallic acid	35	
o-Amino benzoic acid (anthranilic acid)	39	35
Dithiosalicylic acid	39	36
p-Hydroxybenzoic acid	40	30
p-Aminobenzoic acid	40	33
Benzoic acid	40	33
Phenylpropionic acid	40	41*
Acetylsalicylic acid	41	
Benzene sulphonic acid	42	33
Benzylamine	42	38
o-Toluic acid	43	35
m-Phenylene-bis-guanyl guanidine[m]	43	43
Phenylacetic acid	43	46
Mandelic acid	45	45
p-Phenylene diamine	46	42
Nicotinic acid	52	43
Pyridine	52	54
Aniline (p_H 4.5)	53	52
Piperidine	55	53
Benzene disulphonic acid	57	60
Pyrogallol	62	55
Resorcinol	65	61
Isophthalic acid	63	65
Naphthalene 1:5 disulphonic acid[m]	66	63
Salicylsulphonic acid	67	65
o-Phthalic acid	67	70

* The tendon fibres ruptured while attempting to attach them to the lever of the recording apparatus. [m] Tested by the micro-technique and corrected.

Aromatic heterocyclic compounds

The well-known anionic denaturant, salicylic acid, lowered the s.t. more than did any other compound with the exception of phenyl propiolic acid, but it was only slightly more effective than cinnamic acid and gallic acid (Table VII). Benzylamine was the

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most effective of the aromatic cationic compounds tested and it is interesting that its s.t. is close to that produced by guanidine, the best of the aliphatic cationic compounds. Most of the aromatic compounds produced s.t.'s below 45°. Least effective were the heterocyclic compounds, the dibasic aromatic acids, and the poorly ionized aromatic compounds. All the acids which produced average s.t.'s below 53° have pKa values of less than 5*. They would therefore be more than 99% ionized at p_H 7.

Variation in degree of ionization of organic compounds

By increasing the p_H of 1.0 M benzylamine in four steps from 4.3 to 9.7 the s.t. was steadily elevated from 37° to 45°, and by increasing the p_H of 1.0 M aniline in three steps from 4.1 to 6.3 there was a similar elevation of s.t. from 40° to 61°. Since in the absence of salt such changes in p_H would not affect the s.t., it appears that decrease in the ionization of the bases is responsible for the rise observed. The pKa values for benzylamine and aniline are 9.38 and 4.66 respectively at 25°, and they would not differ from these values by more than a fraction of a p_H unit at the s.t.

DISCUSSION

The influence of compounds on the s.t. of collagenous tissues probably depends largely on their degree of adsorption at the collagen-water interface. DOCKING AND HEYMANN[7] have shown that the adsorption of inorganic ions on gelatin conforms with their position in the lyotropic series, and the same order would be expected to hold for their adsorption on the gelatin precursor, collagen. Substances which are strongly adsorbed by gelatin lower the s.t. considerably. If adsorbed only slightly the s.t. may be elevated, particularly if the ion is sufficiently hydrated to withdraw water from the collagen, for drying in air or *in vacuo* produced a considerable rise in s.t. KATZ AND DERKSEN[18] and ASTBURY[1] have shown by X-ray measurements that the side chain spacing in the direction of the salt links increases with hydration from approximately 10 Å to 16 Å for gelatin and to 11.5 Å for collagen. Thus dehydration would bring the adjacent polypeptide chains closer together and increase the opportunity for additional cross linkage between them. Elevation of the s.t. by drying collagen is analogous to the protection against denaturation of globular proteins observed by BEILINSSON[3] when sucrose was incorporated in the denaturant solution.

Amongst the inorganic anions, s.t. lowering varied inversely with hydration. Amongst the fatty acid anions, with the exception of formic acid and acetic acid, s.t. lowering, and presumably adsorption, became more pronounced with increase in the length of the carbon chain. This would be predicted from knowledge that the mutual attraction between organic compounds in solution increases almost linearly with increase in the length of the carbon chain (DUNKEL[8]). The marked influence of many aromatic ions on the s.t. suggests that the possession of a benzene ring is very favourable to adsorption on collagen. Moreover, the introduction of hydrophilic groups, such as OH or NH_2, into the ring of benzoic acid, and preferably in the ortho position, as in salicylic acid and anthranilic acid, promoted s.t. lowering. The favourable influence of such groups on the adsorption of dyestuffs has led to their classification as "auxochrome"

* Dissociation constants of most of the compounds tested are published in "LANDOLT-BÖRNSTEIN, *Physikalisch-Chemische Tabellen*" 5th edit., Julius Springer, Berlin. The others were determined by electrometric titration in the author's laboratory.

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groups. Presumably they promote the s.t. lowering action of ions also by favouring adsorption. The greater effectiveness of amino- and hydroxy-substituted acids than benzoic acid, and the lesser effectiveness of those containing additional saturated hydrocarbon groups, points to the existence of an optimum polar-apolar balance between the head and the tail of the aromatic ion for maximum s.t. lowering.

A second property which largely determines the effect of a compound on the s.t. of collagen, perhaps by influencing its adsorption, is the extent to which it is ionized at the p_H of the experiment (p_H 7.0). Compounds, such as the alcohols, which neither ionize nor react chemically with collagen, had little effect on the s.t. at 1.0 M concentration, and compounds such as urea and formamide, which are only feebly ionized at p_H 7, were less effective than well-ionized compounds. But all compounds which lowered the s.t. by 10° or more are completely ionized at p_H 7 and, if anionic in character, they carry only one negative charge. They probably lower the thermal stability of collagen mainly by competitive adsorption at the salt links. Some polyvalent inorganic or organic anions, such as SO_4^{-2}, PO_4^{-3}, citrate, and those liberated by dicarboxylic acids, elevated the s.t., and amongst the aromatic compounds the divalent anions produced less s.t. lowering than the corresponding univalent anions.

Phthalic acid, for example, produced a higher s.t. than benzoic acid. Possession of more than one negative charge may prevent adsorption at the salt link by reducing the polar-apolar balance, or the divalent anions may elevate the s.t. by forming cross links between the negatively charged groups contributed by the lysine and arginine residues in adjacent polypeptide chains. However, if such cross links were formed the s.t. elevation produced by the various divalent aliphatic anions would not be expected to be so alike regardless of the distance separating the two negative charges on the ions. Moreover, DOCKING AND HEYMANN[7] showed that polyvalent anions were only slightly adsorbed on gelatin. Withdrawal of approximately the same amount of water from collagen by the unadsorbed ions, due to the possession of two negatively charged groups which are equally hydrated regardless of the distance between them, is an alternative explanation. Possession of more than one positive charge on the cation did not render it any less effective in lowering the s.t. than the corresponding univalent cation. Thus, *m*-phenylene-bis-guanyl guanidine lowered the s.t. to about the same extent as guanidine, and Ca, Sr and Ba produced lower s.t.'s than Na, K and Li.

A third property which can make an important contribution to the s.t. lowering activity of an ion is resonance. The lowering of s.t. upon introduction of a double or triple bond into the side chain of an aromatic anion, as in cinnamic acid and phenyl propiolic acid, is probably the result of increased resonance in the ion, thereby enhancing its reactivity with hydrogen bonds between the CO and NH groups of adjacent polypeptide chains in collagen. Such ions could lower the s.t. by breaking both salt links and hydrogen bonds. GREENSTEIN[11] attributed the greater denaturing activity of urea and guanidine on egg white than of certain related compounds to their ability to resonate in aqueous solution. The introduction of halogen atoms into the fatty acid anions, as in bromobutyric acid, iodoacetic and especially in trichloracetic acid, promoted s.t. lowering, yet if the hydrophobic nature of the uncharged portions of the ion were the only consideration the halogenated ions should have lowered the s.t. less than the unsubstituted ions. By attracting electrons within the ion, halogen atoms may increase reactivity with resonating systems in the protein, such as that involving the hydrogen bond. In fact, the hydrogen bond may be the most important site of action for some

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ions. However, saturated and unsubstituted ions such as the higher fatty acid anions, would not be expected to react in this way, and for them competitive adsorption at the salt link is probably the only mechanism.

The greater s.t. lowering of anions, in general, than cations may be due to the excess of lysine and arginine residues, as may be seen from published amino acid analyses of collagen and gelatin (ASTBURY[1]) or alternatively, to the number of amide groups, judging by the liberation of ammonia during the conversion of collagen to gelatin and the accompanying fall in isoelectric point from p_H 7.0 or 7.8 for collagen (HIGHBERGER[13], BEEK AND SOOKNE[2]) to 4.8 for gelatin (HITCHCOCK[14]). An excess of basic groups in collagen would allow penetration of anions into the structure more readily than cations. The explanation of the difference between anions and cations in respect of the influence of their valency on the s.t. will, no doubt, become apparent when more is known of the structure of collagen.

It is difficult to account for the reversal in the relative order of s.t. lowering of skin by inorganic anions with reduction in p_H value below 2.5. Conversion of the skin collagen to the cationic state may be partly responsible, or there may be association of H^+ions with the anions being tested within the skin, thereby lowering the local concentration of both ions. Similar reversal in effectiveness of ions with reduction in p_H value has been demonstrated for other colloidal systems (see, for example, JORDAN LLOYD AND SHORE[17]). Failure to confirm reversal of the lyotropic series when using tendon cannot be explained.

Maximum swelling of tendon fibres and maximum tendency to rupture at about p_H 3.5 suggests that these two effects are related. Both probably depend on the weakening or breaking of entirely different bonds from those which must be broken in order to lower the s.t. It appears that, if the longitudinal strength of the tendon has been weakened by osmotic or other forces, the fibres rupture instead of shrinking when the lateral hydrogen bonds or salt links are broken by heating to the s.t. The greater cross-sectional area and the woven structure of the collagen fibres in the skin specimens would explain their failure to rupture in acid solution.

Long-range elasticity of collagenous tissues was demonstrated in water or in aqueous solution at temperatures above the s.t. The unstabilizing action of 10 M NaCNS on collagen was so pronounced that the s.t. was lowered below 18° and heating was unnecessary. The greater elasticity of the collagen preparation and tendon fibres than skin after heat-shrinking and cooling to 18° suggests that some thermoelastic component was removed from skin during the preparation of collagen, and tendon contains insufficient of such substance to affect its elasticity appreciably. Epidermal keratin was probably the component which restricted the elasticity of skin after cooling.

The influence of ions on physical changes in collagen and gelatin has been the subject of several papers. KATZ AND WEIDINGER[19], for example, showed that the effect of an anion on the s.t. of collagen depends on its position in the lyotropic series, but the effect of cations was not reported. BRAYBROOKS, MCCANDLISH AND ATKIN[4] and THEIS AND STEINHARDT[23] restricted their s.t. studies on cations to Ca, Mg, Na and K. Similar investigations, but dealing with the relationship between the position of an ion in the lyotropic series and the digestion of collagen in salt solutions, were reported by THOMAS AND FORSTER[25], and the importance of the series with respect to the swelling, viscosity, setting and precipitation of gelatin was demonstrated by HOFMEISTER[16], PASCHELES[22], FREUNDLICH AND SEAL[10] and BÜCHNER[5]. BURK[6] drew attention to the lyotropic series of anions

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in relation to the denaturation of globular proteins, as measured by the nitroprusside test for –SH groups.

Studies of the influence of ions on the excitability of muscle (HÖBER[15]), have shown, as in the present paper, that the effectiveness of inorganic ions is related to their position in the lyotropic series and the action of organic ions depends on their hydrophobic-hydrophilic properties. Moreover, like collagen, myosin is converted by guanidine from a fibrous state to one approaching that of the native globular proteins, as evidenced by reduction in viscosity and in double refraction of flow (EDSALL AND MEHL[9]). The mechanism whereby myosin contracts may, therefore, be similar to that suggested for the shrinkage of collagen.

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SUMMARY

The shrinkage temperature (s.t.) of collagenous tissues, such as sheepskin and rat tail tendon, increases with the load applied and with decrease in the moisture content of the tissues. It falls sharply with reduction in p_H value below 4 and with increase beyond 11 and is affected by ions in accordance with their position in the lyotropic series, I^- and CNS^- producing the lowest values.

Unionized organic compounds have little influence on the s.t., but the values produced by the cations of primary aliphatic amines, and by the anions of fatty acids higher in the series than acetic acid, decrease with increase in the length of the C chain when tested at p_H 7. The aromatic anions of phenylpropiolic acid, salicylic acid, cinnamic acid, gallic acid and anthranilic acid lower the s.t. more than any other organic ions tested. Introduction of a second ionized anionic group into an anion represses its s.t. lowering activity, but the introduction of a second ionized cationic group into a cation has no effect.

RÉSUMÉ

La température de rétrécissement (shrinkage temperature ou s.t.) des tissus collagènes, comme la peau de mouton et le tendon de la queue du rat, augmente avec l'augmentation de la charge et avec la diminution du degré d'humidité des tissus.

La chute de la température est remarquable, quand la valeur du p_H tombe au-dessous de 4 ou monte au-dessus de 11; elle est influencée en outre par les ions en conformité avec leur position dans la série lyotropique, I^- et CNS^- produisant les valeurs les plus basses.

Les combinaisons chimiques non-ionisées n'influencent que légèrement la s.t., tandis que les valeurs produites par les cations des amines aliphatiques primaires et par les anions des acides gras, plus hauts dans la série que l'acide acétique, diminuent avec l'augmentation de la longueur de la chaîne C, quand éprouvées à p_H 7. Les anions aromatiques de l'acide phénylpropiolique, de l'acide salicylique, de l'acide cinnamique, de l'acide gallique et de l'acide anthranilique réduisent la valeur de la s.t. plus que tous les autres ions organiques éprouvés. L'introduction dans un anion d'un second groupe ionisé d'anions réprime son influence réduisante sur la s.t., tandis que l'introduction dans un cation d'un second groupe ionisé de cations n'a pas d'effet.

ZUSAMMENFASSUNG

Die Schrumpfungstemperatur (S.t.) kollagener Gewebe, wie Schafshaut und Rattenschwanzsehne, nimmt mit der angewandten Belastung und mit der Verringerung des Feuchtigkeitsgehaltes

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der Gewebe zu. Bei Verminderung des p_H-Wertes unter 4 und Erhöhung über 11 fällt sie scharf ab; durch Ionen wird sie in Übereinstimmung mit deren Platz in der lyotropen Reihe beeinflusst, wobei I^- und CNS^- die niedrigsten Werte verursachen.

Nicht ionisierte organische Verbindungen beeinflussen die S.t. nur in geringem Masse, aber die Werte, die durch die Kationen primärer aliphatischer Amine und durch die Anionen von Fettsäuren, die in der Reihe höher stehen als Essigsäure, entstehen, nehmen bei Zunahme der Länge der Kohlenstoffkette ab, wenn sie bei p_H 7 bestimmt werden. Die aromatischen Anionen von Phenylpropionsäure, Salicylsäure, Zimtsäure, Gallussäure und Anthranilsäure verringern die S.t. mehr als alle anderen untersuchten organischen Ionen. Einführung einer zweiten ionisierten Aniongruppe in ein Anion unterdrückt seine S.t.-erniedrigende Aktivität, während die Einführung einer zweiten ionisierten Kationgruppe in ein Kation keinen Effekt hat.

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