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Rate of Shrinkage of Tendon Collagen: Further Effects of Tannage and Liquid Environment on the Activation Constants of Shrinkage

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Further studies of the rate of shrinkage of tendon collagen are reported. Metallic tannages decreased heat and entropy of activation. Three organic tannages yield evidence of cross-linking by increasing heat of activation. Effects of aqueous solutions of salts of alkali and alkaline earth metals and organic materials are studied. The results—decreased heat and entropy with increasing concentration of alkali salts and a similar behavior with organic solutes over a portion of the concentration range—are interpreted as due to interaction of solutes at hydrogen bonds. Solutions of alkaline earth salts increase heat and entropy of activation. A mechanism of shrinkage designed to explain the results of these studies is proposed. Applications of the experimental method are discussed.

I. Introduction

This investigation represents a continuation of studies of the effects of tannage and environment on the rate of shrinkage of tendon collagen and on the heat, entropy, and free energy of activation of the shrinkage process. It was shown that shrinkage of collagen could be treated as a rate process [1],[1] and that tanning or treatment with sodium chloride, sulfuric acid, and sodium hydroxide generally decreases the heat and entropy of activation. Chrome tanning, however, was shown to cause a marked increase in all activation constants. As a result of the foregoing studies, it was concluded that the various tannages and ionic media investigated interacted principally at salt bonds, possibly causing rupture of these bonds. Chrome tanning was assumed to form cross linkages or to reinforce existing cross linkages.

This report contains further data on the effect of tannage and shows that increased activation constants result from tannages such as quinone and glyoxal, which might be expected to form cross linkages. The effect of environment is studied in greater detail with aqueous solutions of organic solutes and additional inorganic salts being investigated. It is shown that, inasmuch as organic solutes also decrease the activation constants, this decrease may not be attributed specifically to salt-bond interactions of the solute. The behavior of tendon collagen in aqueous solutions is indicative of an extremely complex system, and, in general, no interpretation of the data obtained with solutions is offered.

[1] Figures in brackets indicate the literature references at the end of this paper.

II. Method of Measurement and Treatment of Results

The experimental details of the measurements, as well as the method of analysis of the data, have been described previously [1] and will be treated only briefly here.

Measurements of rate of shrinkage of kangaroo-tail tendon were made at approximately four different temperatures spaced 2 deg to 3 deg C apart, each temperature of measurement being maintained to within ± 0.1 deg C by means of a temperature-controlled water bath. The data consisting of length and time measurements were interpolated to obtain the time of half shrinkage designated as $t_{1/2}$ and defined as the time at which the length of

tendon, l, is one-half of the sum of the initial length, l_0, and the final length, l_∞.

Experimental values of $t_{1/2}$, in seconds, and the reciprocal of the absolute temperature, $1/T$, are introduced into the equation,

$$\log \frac{0.693h}{kTt_{1/2}} = \frac{-\Delta H}{2.303R} \times \frac{1}{T} + \frac{\Delta S}{2.303R}. \quad (1)$$

In eq 1, h is Planck's constant; k, Boltzmann's constant; ΔH, the heat of activation; ΔS, the entropy of activation; and R, the gas constant. This equation is a result of the theory of absolute reaction rates [2] and the utilization of $t_{1/2}$ in place of the reaction-velocity constant. A discussion of this substitution has been given previously [1]. A plot of the left term as ordinate and $1/T$ as abscissa yields a line having a slope proportional to ΔH and an intercept proportional to ΔS. Values of ΔH and ΔS are calculated from the data by the method of least squares and are used in conjunction with the well-known relationship,

$$\Delta F = \Delta H - T\Delta S, \quad (2)$$

to determine ΔF, the free energy of activation. Since ΔF varies with temperature, the temperature for which the calculation was made is denoted by a subscript.

Since ΔF is directly related to the rate of shrinkage, the value of ΔF_{60} may be taken as a measure of the temperature range in which shrinkage occurs at an experimentally measurable rate. It should be noted that a value of ΔF of the order of 25 kcal corresponds to a shrinkage half-time of the order of 1 minute. For purposes of comparison, there are tabulated values of the shrinkage temperature, T_s, obtained directly from the experimental data and defined as the temperature at which half-shrinkage occurs in 1 minute.

From the effect of treatment and environment on the heat of activation, ΔH, calculated from the theory of absolute reaction rates—or the energy of activation E in the Arrhenius expression [2], which is approximately the same numerically—conclusions have been drawn concerning the structure of collagen, the mechanism of tanning, and the mechanism of shrinkage. Some conclusions have similarly been drawn from variations in values of ΔF_{60}. In order to arrive at such conclusions as to the effect of treatment, it has been necessary to assume that the size of the unit taking part in the shrinkage process is unaffected by treatment.

Three general types of results have been obtained in these experiments, namely: ΔH and ΔS decrease while ΔF_{60} increases (inorganic tannages); ΔH, ΔS, and ΔF_{60} increase (deaminization and postulated organic cross-linking tannages); no simple correlation exists between ΔH, ΔS, and ΔF_{60} (variation of environment with organic and inorganic solutes and chemical modifications excluding deaminization). The data are divided into these three general categories, although for purposes of comparison the separation is not adhered to rigorously in some instances.

A more detailed exposition of the mechanism of shrinkage as related to the activation process is given in section IV.

III. Results and Discussion

1. Tannage With Inorganic Materials

Tendons were tanned with several inorganic materials in addition to those previously described [1]. Tannage was effected by immersing the tendons in solutions of the inorganic salts and adjusting the pH of the solutions with acid or alkali to the optimum value. After a 24-hour immersion tendons were removed, washed thoroughly, and stored in distilled water at pH6. The results of tests on these specimens are given in table 1.

By comparison of the values of ΔH, ΔS, and ΔF_{60} for the tanned specimens with those for untreated tendon, which were obtained previously [1] and are included for reference, it is seen that ΔH and ΔS values are lowered whereas ΔF_{60} values are, in general, increased on tannage. Consideration of the standard deviations of the measurements indicates that most changes are too small to be considered significant, except insofar as ΔF_{60} values of copper and mercury tannages are concerned. Since ΔF_{60} is a measure of the "shrinkage temperature" [1], it is seen that most shrinkage temperatures were increased as a result of the tannages.

It will be noted that little correlation exists between the values of ΔF_{60} and the ash content, considered to be a measure of the amount of combined material. Ash content of mercury tannage was not obtained, but wet digestion and precipitation with H_2S showed qualitatively that a small amount of mercury was present.

TABLE 1. *Effect of tannage with inorganic tanning materials*

Tannage	Tanning material	Ash (dry basis)	ΔH	ΔS	ΔF_{60}
		Percent	*kcal/mole*	*cal/mole deg*	*kcal/mole*
Tungsten	Sodium tungstate [3]	30.9	110	253	25.5
Molybdenum	Sodium molybdate [3]	15.0	119	284	24.7
Phosphate	Calgon [3]	1.1	129	311	25.5
Copper	Ammoniacal copper sulfate	3.1	141	340	28.0
Mercury	Mercuric chloride	------	120	276	28.7
Untreated tendon [1]	------	------	141	349	24.7
Standard deviation of value obtained on untreated tendon [1].	------	------	15	43	0.6

As far as can be ascertained, the effects of copper and mercury are being reported for the first time. Both materials appear to tan if elevation of the shrinkage temperature is considered a criterion of tannage. Copper interacts with collagen rapidly and readily in solutions containing both cuprammonia ion and undissolved cupric hydroxide. Mercury reacts from solutions of mercuric chloride having a pH close to the precipitation point. Extensive studies of these reactions have not been made, but hide squares have been subjected to the treatments. Copper appears to produce good "leathering," but mercury treatment appears to resemble "tawing." Both reactions appear to be somewhat reversible.

There is no indication from the results of tannage with inorganic materials that any metallic tannage investigated resembles chrome tannage, which produces an elevation of 80 percent or more in the heat, entropy, and free energy of activation.

2. Cross-Linking Tannages and Treatments

In a previous investigation [1] it was concluded that chrome tanning either formed cross-linkages between neighboring polypeptide chains or reinforced existing linkages. This conclusion was based on the fact that chrome tanning caused an increase in ΔH, which was not found with any other tannage then studied. Three other tannages have been observed to cause an increase in ΔH, namely, quinone, glyoxal, and cyclohexane disulfonyl chloride, as well as treatment designed to deaminize the parent collagen. The results of tests on specimens subjected to these treatments are given in table 2, and graphs representing a portion of these data are shown in figure 1.

Tannages with cyclohexane sulfoynyl chlorides were made in a two-phase system consisting of a solution of the sulfonyl chloride in cyclohexanone and an aqueous solution of sodium bicarbonate. Tannage required several weeks, being considered satisfactory when specimens immersed in water at room temperature showed no indication of putrefaction in 1 week.

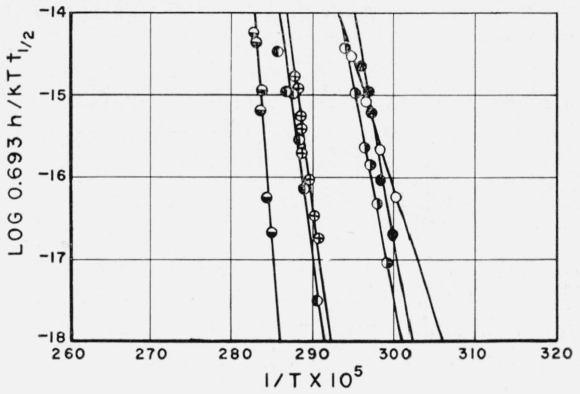

FIGURE 1. *Effect of postulated cross-linking agents on rate of shrinkage.*

○, Cyclohexane monosulfonyl chloride tanned; ◑, cyclohexane disulfonyl chloride tanned; ⊕, glyoxal tanned; ◐, quinone tanned; ●, deaminized 72 hours, pH 6.4; ◒, deaminized 72 hours, chrome tanned.

TABLE 2. *Effect of cross-linking treatments*

Treatment	ΔH	ΔS	ΔF_{60}
	kcal/mole	*cal/mole deg*	*kcal/mole*
Tanned with cyclohexane monosulfonyl chloride [4]	145	361	24.9
Tanned with cyclohexane disulfonyl chloride [4]	217	573	26.4
Tanned with glyoxal at pH 5.2	288	760	34.6
Tanned with quinone at pH 4.0	271	710	35.9
Deaminized 24 hours at 0° C, tested at pH 5.5, set A	185	477	26.3
Deaminized 72 hours at 0° C:			
Tested at pH 6.4, set A	230	613	25.7
Tested at pH 4.3, set A	191	493	26.5
Deaminized 72 hours at 0° C and chrome-tanned (1.1% Cr_2O_3), set A	456	1,225	48.4
Deaminized 48 hours at 0° C, set B	256	701	22.7
Deaminized 48 hours at 0° C and glyoxal-tanned at pH 5.2, set B	313	877	20.8
Deaminized 48 hours at 0° C and quinone-tanned at pH 5.0, set B	300	827	24.5
Tanned with chromium [1] (1.02% Cr_2O_3)	390	1,041	44.1

Tannage with glyoxal was carried out in a 5-percent aqueous solution of glyoxal at pH 5.2 for 24 hours.

Tannage with quinone was conducted in a saturated aqueous solution of quinhydrone at pH 4.0 for 24 hours.

Deaminization was carried out according to the procedure of Highberger [5]. Deaminized tendons were chrome-tanned by the two-bath process described previously [1]. Deaminization was performed in two separate experiments, the first of which was designed to ascertain the effect of deaminization on chrome tanning. Results from measurements on this sample are designated as set A. The second experiment was designed to determine the effect of deaminization on glyoxal and quinone tannage, and results of this experiment are designated as set B.

The results shown in table 2 show that tannage with the monosulfonyl chloride yields normal values of ΔH, ΔS, and ΔF_{60}. However, if tannage is carried out with the disulfonyl chloride, it is observed that ΔH, ΔS, and ΔF are increased significantly. The cyclohexane monosulfonyl chloride tannage has been investigated by Patterson [4] who concluded that the sulfonyl chloride reacts with amino groups present in the collagen, the reaction proceeding with liberation of HCl as follows:

$$R'-SO_2Cl+H_2N-R\rightarrow R'-SO_2HN-R+HCl, \quad (3)$$

where R′ represents a cyclohexane group and R a peptide chain. Similarly, a disulfonyl chloride may be expected to link two amino groups as follows:

$$R-NH_2+ClSO_2-R'-SO_2Cl+H_2N-R\rightarrow 2HCl+R-NHSO_2R'\ SO_2HN-R. \quad (4)$$

Since collagen contains amino groups in excess of those involved in peptide linkages [6], reaction 4 might be expected to proceed between pairs of amino groups in the required spatial configuration. This reaction might form an external bridge along the peptide chain or a cross-linkage between peptide chains. The fact that an increased value of ΔH results from this tannage supports eq 4, since such linkages would presumably be broken in the activation process and therefore larger ΔH values would be required. The compound formed in eq 3 would not result in increased ΔH values, as the linkage formed would not be expected to be critical from the point of view of activation.

It has been found [1] that tannage with formaldehyde did not increase ΔH, which was taken to indicate that cross-linking did not occur in this tannage [7]. However, it might be expected that a dialdehyde would cross-link two free amino groups, and tests made with glyoxal-tanned specimens show large increases of ΔH consistent with this view. The same result was obtained with quinone tannage as shown in table 2. It will be noted that glyoxal and quinone produced approximately twice as great an *increase* in ΔH as the disulfonyl chloride. Inasmuch as the extent of the reactions is not known, no conclusions may be drawn from this result. The proposed reactions involved between collagen and aldehydes and quinone have been outlined in detail [3] and will not be discussed here.

It is noted that the results obtained from the two deaminization experiments—sets A and B—agree as regards effect of deaminization on ΔH and ΔS, but not on ΔF_{60}. The reason for this discrepancy is not known but may be attributed to differences in treatment subsequent to deaminization. At the conclusion of deaminization and thorough washing in *cold* water, the specimens acquire a golden yellow color. If tested immediately, it is found that the warm water required for shrinkage causes an apparent elevation of the temperature required for shrinkage and simultaneously a deepening of the color of the specimens. If deaminized specimens stand in water at room temperature, the color likewise deepens to a reddish brown. Set A was tested several weeks after deaminization, whereas set B was tested within a few days after preparation. The difference in time of standing may be the cause of the discrepancy in ΔF_{60} values. The significant increase in ΔH values found on deaminization may be interpreted to mean that the interaction of nitrous acid with collagen produces some degree of cross-linking between peptide chains.

Stubbings and Theis [8] have found that the ϵ-amino group of lysine in collagen is destroyed rapidly during deaminization, but that the arginine residue is attacked slowly. The ϵ-amino group of lysine has little influence on chrome tanning, as shown by the 250 kcal/mole *increase* in ΔH caused by 1.1 percent of Cr_2O_3 in the deaminized specimens. This value agrees with

the *increase* of 240 kcal/mole in ΔH caused by 1.0 percent of Cr_2O_3 in untreated specimens [1]. These results imply that the ϵ-amino group of lysine is not primarily involved in chrome tanning.

Glyoxal and quinone tannages cause increase in ΔH values of deaminized specimens. This increase is consistent with postulated cross-linking due to the tannages. However, the *increase* in ΔH observed on tanning deaminized specimens is less than half that obtained on tanning untreated specimens. This finding probably indicates that the ϵ-amino group of lysine is involved in the reactions with glyoxal and quinone, but that reaction also occurs at other groups—possibly at the arginine residue not removed in deaminization. It appears that cross-linking caused by glyoxal and quinone takes place, partly at least, between the ϵ-amino groups of lysine as expected.

3. Chemical Modifications

In order to ascertain the effect of certain treatments on the collagen molecule, tests were made on chemically modified collagen. It must be emphasized that the results of such tests are not liable to strict interpretation because of the complexity of collagen and the lack of detailed information on specific changes caused in the molecule. The following treatments were investigated.

(a) Dry Heat

A sample of air-dried tendon collagen was heated for 48 hours at 50° C, for 72 hours at 100° C, for 72 hours at 120° C, and for 72 hours at 150° C. At the conclusion of the heating period at each temperature a specimen of suitable size was removed, soaked in water for 72 hours, and tested. It is to be noted that a single sample was carried through the heating cycle so that the specimen, removed at 150° C, had been subjected to temperatures of 50°, 100°, and 120° C for the periods specified.

(b) Esterification

Esterification of free carboxyl groups was attempted by the method described by Fraenkel-Conrat and Olcott [9] using methyl alcohol. The reaction was carried out for 4 days at room temperature. A portion of the specimens was washed free of acid in a salt solution, while the remainder was subjected to the action of concentrated ammonium hydroxide for 24 hours, washed, and tested.

(c) Methyl Magnesium Iodide

Vacuum-dried tendons were subjected to the action of methyl magnesium iodide at 30° C for 1 week. Possible reaction products were hydrolyzed in a pickle solution at 0° C, and the tendons were washed free of acid and salt, and tested.

The results of tests of specimens subjected to the foregoing treatments are given in table 3.

TABLE 3. *Effect of chemical modifications*

Treatments	ΔH	ΔS	ΔF_{60}
	kcal/mole	*cal/mole deg*	*kcal/mole*
Heated at 50° C for 48 hr	165	422	24.9
Heated at 50° C for 48 hr and at 100° C for 72 hr	136	340	23.1
Heated at 50° C for 48 hr, at 100° C for 72 hr, and at 120° C for 72 hr	88	204	20.1
Heated at 50° C for 48 hr, at 100° C for 72 hr, at 120° C for 72 hr, and at 150° C for 72 hr	[a]	[a]	[a]
Methyl magnesium iodide	148	375	23.7
Esterified with methyl alcohol, washed	[a]	[a]	[a]
Esterified with methyl alcohol—subsequent ammonia treatment	160	427	17.5

[a] No measurements possible.

It is observed that dry heat appears to deteriorate collagen since ΔH, ΔS, and ΔF_{60} values appear to decrease monotonically. Results obtained on specimens heated at 50° and 100° C, however, are not significantly different from those obtained on untreated material. After heating at 120° C, a drastic, significant lowering is found, whereas specimens subjected to heating at 150° C shrank and disintegrated on soaking so that no measurements could be made. Kanagy [10] has shown that carbon dioxide and water are liberated from leather heated in dry air and oxygen and concludes that oxidation may be occurring at temperatures as low as 80° C. This oxidation may be the cause of the rapid decrease in cohesive forces shown by the figures of table 3.

Treatment with methyl magnesium iodide produced no visible reaction, and the results obtained are normal. Since the Grignard reagent is a severe dehydrating agent, it would appear likely that the results obtained on dry heating are due to the effect of heating and not merely to loss of water.

Esterification produced a product that appeared unstable in water, and no measurements were made

on these specimens. Following esterification, specimens were washed in a salt solution for several weeks until the pH of the solution showed no decrease on standing in contact with the specimens for 24 hours. However, on replacing the salt solution with water, extreme swelling occurred, and it was not possible to perform measurements on the specimens. The cause of this swelling is not known, but previous studies have also shown swelling as a consequence of esterification [11].

Specimens subjected to treatment with a solution of concentrated ammonia after esterification, in expectation of replacing the ester groups with amide groups, appeared to be stable in water, but yielded low ΔF_{60} values.

4. Effect of Changing Test Medium

(a) Special Considerations of Data on Effect of Test Medium

Results obtained on tanned tendons appear much easier to interpret than data concerning the effect of variation of the test medium. In tanning tests, the data for tanned tendons are compared with those for untanned specimens tested under the same conditions of environment. When the medium is changed, comparison of results with those of a standard environment (i. e., water, pH 6 to 7, 60° C) may lead to conclusions that are erroneous, because the chemical potential of the test medium is changed by addition of solute. The chemical potentials of both solvent and solute may be involved in the activation process in an unknown manner. Eyring and Stearn [12] and Neurath et al. [13] discuss this point in detail. It appears, therefore, that it is of questionable value to attempt the interpretation of the data obtained with aqueous solutions.

(b) Neutral Salts of Alkali Earth Metals

Previous experiments [1] on the effect of sodium chloride solutions yielded results that were interpreted as the result of solute ions interacting at ionic linkages in the collagen. In order to ascertain the effect of other salts, measurements were made using aqueous solutions of a variety of neutral salts of various concentrations. The results obtained for salts of alkali metals are given in table 4.

The similarity of the general nature of the results obtained with sodium nitrate and potassium chloride solutions to those previously reported for sodium chloride is to be noted. Sodium sulfate solutions produce somewhat different effects, while lithium chloride and sodium thiocyanate solutions produce drastic lowering of T_s values.

TABLE 4. *Effect of solutions of salts of alkali metals*

Solute	Concentration	pH	ΔH	ΔS	ΔF_{60}	T_s
	Mole/liter H_2O		*kcal/mole*	*cal/mole deg*	*kcal/mole*	° *C*
$NaNO_3$	0.02	6.8	152	384	23.7	63
Do	.5	6.3	125	311	20.9	55
Do	3.0	5.5	97	233	19.6	50
Do	7.0	------	110	260	23.0	61
Do	[a]	6.4	90	200	23.8	64
Na_2SO_4	0.10	------	139	322	22.7	61
Do	.25	------	116	278	23.6	63
Do	.5	------	96	214	24.7	68
Do	1.0	------	106	232	28.4	79
NaSCN	0.1	5.5	133	332	22.8	61
Do	.5	6.0	136	356	17.7	49
Do	1.0	------	123	327	14.0	40
KCl	0.1	6.4	114	274	23.2	61
Do	.5	6.3	124	307	22.1	59
Do	1.0	6.1	103	241	23.1	61
Do	3.8	5.7	101	225	26.4	74
LiCl	1.0	6.5	135	349	19.1	52
Do	2.5	------	132	347	16.4	45
Do	3.5	6.4	128	335	16.6	40
Do	5.0	6.8	121	335	9.6	27

[a] Saturated at 20° C.

C. Neutral Salts of Alkaline Earth Metals

The drastic action of alkaline earth chlorides is well known [14], and it is of interest to ascertain the effect of solutions of such salts on the activation process. Studies were confined to the chlorides of barium, calcium, and magnesium, and the sulfate of magnesium. The latter is of particular interest since it is widely used as a filler in sole leathers. The results of these tests are shown in table 5.

The similarity of the results obtained with the chloride solutions is marked as is the difference between the effects of chloride and sulfate of magnesium. The divergence of the data obtained with solutions of magnesium chloride and magnesium sulfate is shown graphically in figure 2.

It was not possible to pursue investigations of solutions of alkaline earth chlorides to higher concentrations, as shrinkage occurs at or below room temperature in such solutions. Some of the results reported necessitated measurements below the ambient temperature. However, to resolve the

question of a minimum shrinkage temperature with these salts, observations were made in every instance on saturated solutions of the chlorides. It was found that shrinkage occurred very slowly at discrete regions of the specimens below 20° C in all saturated solutions, which shows that there is no minimum on these curves corresponding to the minimum shrinkage temperature observed for solutions of the alkali chlorides.

TABLE 5. *Effect of solutions of salts of alkaline earth metals*

Solute	Concentration	pH	ΔH	ΔS	ΔF_{60}	T_s
	Mole/liter H_2O		*kcal/mole*	*cal/mole deg*	*kcal/mole*	° *C*
$BaCl_2$	0.10	6.1	137	347	21.8	59
Do	.25	6.1	215	594	17.0	52
Do	.50	6.1	206	578	13.5	46
Do	1.00	5.9	175	494	10.3	38
$CaCl_2$	.10	6.6	136	347	20.9	56
Do	.25	6.6	160	424	18.7	52
Do	.50	6.2	140	372	16.2	46
Do	.75	6.0	230	667	7.7	40
$MgCl_2$	.02	6.1	110	263	22.8	61
Do	.10	6.1	132	334	20.9	57
Do	.25	6.0	139	356	20.3	55
Do	1.00	------	191	540	11.4	42
Do	1.40	------	210	601	10.3	41
$MgSO_4$	.10	------	164	424	22.4	60
Do	.50	------	122	297	22.8	61
Do	1.00	------	134	326	25.3	67
Do	1.70	------	121	274	30.2	81
Do	2.20	------	139	317	33.9	91
Do	[a]	------	119	257	33.4	92

[a] Saturated at 20° C.

(d) Solutions of Organic Solutes

The previous conclusion that salts interacted principally at salt-bonds was tested by subjecting specimens to test media of aqueous solutions of organic materials. Such experiments were also expected to yield information on the role of water in the shrinkage process. The complete results of these tests are given in table 6 and for ethyl alcohol and dioxane in figure 3.

The results given in table 6 show that, except for glycerine, the organic solutes, in general, behave in a similar manner to the alkali chlorides in dilute solutions. In more concentrated solutions, however, all quantities increase significantly. If ionic linkages exist in collagen, and are involved in the shrinkage process, it would be expected that ΔH should increase by virtue of the lowering of the dielectric constant in these solutions and the increased cohesive energy of the collagen

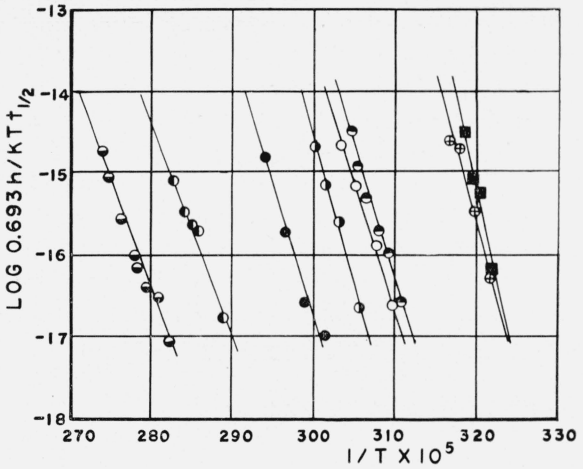

FIGURE 2. *Effect of magnesium chloride and magnesium sulfate solutions on rate of shrinkage.*

◑, $MgSO_4$, 0.1-M solution; ●, $MgSO_4$, 1-M solution; ◐, $MgSO_4$, 1.70-M solution; ◒, $MgSO_4$, saturated solution; ○, $MgCl_2$ 0.1-M solution; ◓, $MgCl_2$, 0.25-M solution; ⊗, $MgCl_2$, 1-M solution; ▩, $MgCl_2$ 1.4-M solution.

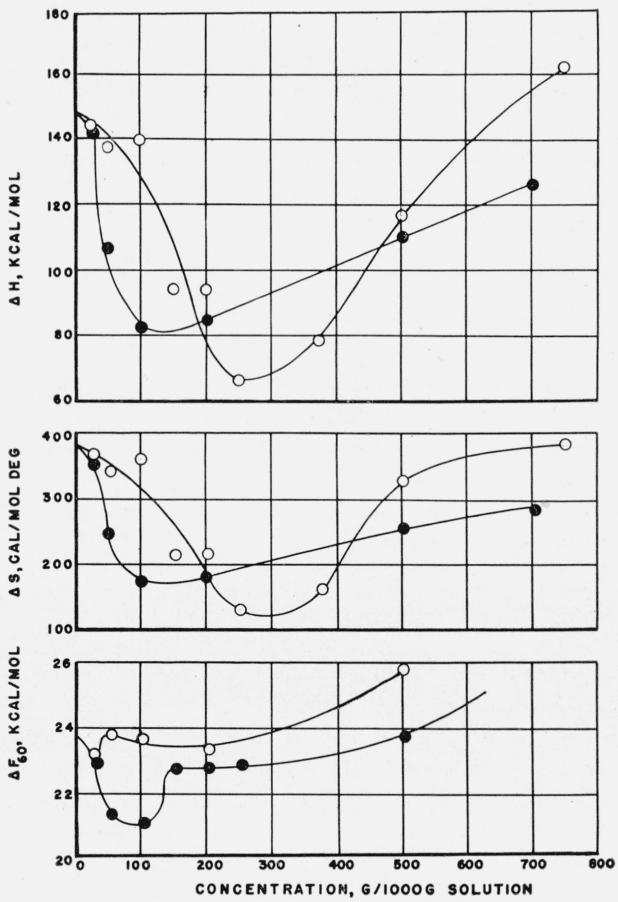

FIGURE 3. *Heat, entropy, and free energy of activation in aqueous solutions of alcohol and dioxane.*

●, Alcohol solutions; ○, dioxane solutions.

molecule. The reverse effect was observed, and it appears that ionic linkages are probably not involved in the shrinkage process, and furthermore that effects produced by solutions of alkali salts are probably not the result of interactions of ions at such linkages. The alternative explanation of this effect [1] that the results obtained are due to interactions of the various solutes at hydrogen bonds existing in the collagen is, therefore, proposed as more in accord with the experimental facts.

TABLE 6. *Effect of aqueous solutions of organic materials*

Solute	Concentration	ΔH	ΔS	ΔF_{60}	T_s
	g/1,000 g solution	*kcal/mole*	*cal/mole deg*	*kcal/mole*	*° C*
Dioxane	25	145	367	23.2	62
Do	50	136	343	21.4	59
Do	100	141	360	21.1	57
Do	150	95.1	217	22.8	60
Do	200	94.4	215	22.8	60
Do	250	66.5	131	22.9	60
Do	370	78.7	165	23.8	64
Do	500	137	328	27.5	74
Do	750	162	384	34.7	87
Ethyl alcohol (95%)	25	144	364	23.0	61
Do	50	107	249	23.9	64
Do	100	83	178	23.7	62
Do	200	85	184	23.4	63
Do	500	110	256	25.8	71
Do	700	127	287	30.9	[a] 86
Glycerine	25	122	295	23.9	64
Do	100	129	315	23.7	62
Do	170	137	342	22.7	61
Do	250	189	510	19.6	54
Do	500	225	622	18.2	53
Do	921	56	96	24.3	70
Glucose	25	152	383	24.5	64
Do	50	120	290	23.9	64
Do	100	122	298	23.1	61
Do	200	147	374	22.1	58
Do	400	144	370	21.3	56
Do	700	151	388	21.8	59

[a] This value obtained by extrapolation of experimental data.

The results obtained with glycerine differ from those obtained with the other organic solutes. Values of ΔF_{60} show that a decrease in shrinkage temperature takes place in concentrated glycerine solutions such as are used in testing shrinkage temperature [13]. This result suggests that the use of glycerine for such measurements is not desirable. It would seem that when shrinkage temperatures exceeding 100° C are to be measured, the use of water under pressure should yield more reliable results. Results obtained in solutions containing 97.9 percent of glycerine were not obtained under the same experimental conditions as other results and are, therefore, not to be compared with the other data. It was observed that this concentrated glycerine solution penetrated tendon collagen slowly, and the results shown were obtained on specimens soaked in glycerine for 30 days. The low heat of activation obtained, together with the observed swelling in glycerine, indicate a deterioration of collagen by glycerine. This finding emphasizes the fact that all results reported must be considered as "short term" effects only.

IV. Mechanism of Shrinkage of Collagen

Many qualitative descriptions of the mechanism of shrinkage of collagen have been published [16, 17]. Theories that have been proposed are in substantial agreement, differing only in minor aspects. As a result of the present studies, a qualitative mechanism has been evolved that is in general agreement with those previously reported. However, details of the shrinkage process, which have not been considered in any previous report, are vital in this mechanism and will be discussed at length. The proposed process is as follows:

Collagen may be pictured as containing regions of varying degrees of order. The structure of the highly ordered regions of parent collagen has been studied extensively by means of X-ray diffraction [18, 19]. Results of such studies are interpreted to indicate a grid-like network of polypeptide chains cross-linked by hydrogen bonds and separated by a distance of 4.4 A. Neighboring grids are separated approximately 11 A by side chains, which link the grids to each other.

A general picture of the shrinkage process may be described as follows: It might be expected that over some elevated temperature range *dry* collagen would become amorphous [20], but decomposition occurs before these temperatures are attained [21]. However, upon immersing collagen in an aqueous medium, water penetrates between the grids of chains, increasing the spacing irregularly from 11 A to at least 17 A [18]. If this system is heated, the increase in kinetic energy due to the combined thermal agitation of the polypeptide chains *and the imbibed water* becomes sufficient at some elevated temperature

to rupture the stabilizing, cross-linking bonds. The unsupported chains then spontaneously undergo disorientation to a folded configuration of higher entropy. In the folded configuration, the chains occupy a greater volume [22] and exhibit no evidence of long-range periodicity [19]. The swollen, thermally contracted collagen is rubber-like and probably contains few, if any, cross-linkages between peptide chains. The term "cross-linkage," as used here, refers to any lateral bonding through primary or secondary valence forces.

The foregoing picture is well known and generally accepted. However, this mechanism does not account either for the inhomogeneous nature of the shrinkage process reported in a previous paper [1], and which has been shown to occur even in submicroscopic fibrils [23], or for the time dependence of the phenomenon. In order to explain this behavior, the following detailed mechanism of the shrinkage process is proposed:

Inasmuch as variations in chain spacing, spatial orientation, and chemical structure probably exist in pure collagen, it is not likely that all cross linkages possess equal energies, or even that extremely small, arbitrary regions contain equal numbers of such linkages. A small region containing few linkages of low energy is considered to represent a site favorable for initiation of shrinkage, i. e., a "shrinkage nucleus."

Consider a region containing such a nucleus. The nucleus is assumed to contain a large number of peptide chains and their accompanying cross-linkages. Due to kinetic energy of thermal agitation and thermal bombardment by water molecules, any particular cross linkage may be broken and a degree of freedom thereby imparted to two chains even at a low temperature. Upon loss of the linkage, the sites of the former bond are assumed to be saturated with water molecules made available by motion of the chains and held by secondary chemical forces. Barring further developments, the water molecules attached to the chains may be ejected by agitation of the partially free chains, and recombination of the original bond may thus occur after a suitable interval. Shrinkage of a pair of chains freed in this manner is not likely to occur because of the rigidity conferred by neighboring chains that are still cross-linked. As the temperature is raised, more energy is available for bond rupture, and the number of bonds broken per unit time becomes larger. The chains freed by the ruptures move about, combine with water, subsequently recombine, etc.

It is assumed that when some large minimum number of bonds in this nucleus are open at the same instant, the structure is so weakened that there exists a very high probability that the nucleus will proceed to a more stable configuration (the shrunken state) by chain folding. For this process it is believed cooperation between many chains is required, therefore many chains shrink simultaneously. The configuration in which the requisite number of bonds are open is considered to be the activated complex [1].

These shrinkage nuclei may occur at random locations throughout the collagen, and shrinkage initiated in such nuclei may possibly occur at any time. However, it is obvious the process is both temperature and time dependent—a rate process—although the probability of such occurrence at low temperature becomes prohibitively small. The nodules of initiation of shrinkage previously described [1] are ascribed to shrinkage occurring at these shrinkage nuclei.

Following the formation of a single shrunken nucleus, intact bonds existing between chains in the immediate vicinity are most probably subjected to higher forces imposed by loss of reinforcing bonds that existed in the shrunken nucleus and the distortion caused by this material. The shrunken nucleus may be expected to grow, therefore, both laterally and longitudinally at the expense of the chains in these directions.

In the lateral directions the nucleus is expected to grow rapidly, as smaller numbers of chains in these directions may collapse as a unit. This rapid lateral growth is believed to result in the gelatinous rings previously described [1]. Longitudinal growth will take place more slowly, because the intact lateral structure in these directions will necessitate cooperation between more chains, and therefore simultaneous shrinkage of a large number of chains. The processes occurring in the regions longitudinally adjacent to the shrunken nucleus are, therefore, pictured as very similar to those occurring in the "shrinkage nucleus" itself, and shrinkage will take place only after a large number of bonds are open simultaneously.

Longitudinal shrinkage probably occurs at a somewhat higher rate than initiation of shrinkage,

as bonds in the longitudinally adjacent regions are probably subjected to larger forces arising from the deformation and freedom of motion of chains in the shrunken nucleus. The longitudinal growth, however, will probably exhibit essentially the same time and temperature dependence as the formation of the shrunken nuclei. This assumption is justified by linearity of rate data over the temperature range studied, although an exception has been noted [1] in the case of highly swollen tendons in which large numbers of bonds are probably broken before testing, or in which smaller units may shrink because of the swollen condition of the collagen.

The energy absorbed by the shrinkage nucleus and the corresponding longitudinally adjacent region in the transformation from the condition in which the cross linkages are intact to that configuration in which the requisite minimum number for shrinkage are broken, is the heat of activation, ΔH. For the reasons given previously, it is believed that ΔH is essentially the same for both the initiation and propagation of shrinkage. The values of ΔH obtained in these studies are probably an average of two very large, nearly identical values corresponding to the initiation and the propagation of shrinkage.

The shrunken collagen in the wet condition will be rubber-like, as available cross-linking sites are combined with water molecules. On partial or complete drying, cross linkages will be reformed between active sites of adjacent folded chains. The dry, or partially dry, shrunken collagen, therefore, will be rigid despite the folded configuration of the chains.

The bonds that cross-link the peptide chains of parent collagen and which are involved in the foregoing mechanism have not been identified. The nature of these bonds does not affect the proposed process. From these studies it is concluded that the *only* bonds involved in the process are the hydrogen bonds existing between laterally adjacent peptide linkages. This conclusion has not been reached by virtue of direct evidence of the participation of hydrogen bonds, but by considerations and data pointing to the nonparticipation of salt bonds. The reasons for rejecting the concept that salt bonds are involved in shrinkage are as follows:

The number of such linkages is probably small because of the spatial requirements involved. Hydrogen bonds may be estimated to possibly outnumber salt bonds by a factor of seven.

Salt bonds existing in an aqueous medium of the type used in shrinkage measurements are expected to be labile.

Deaminization of collagen does not produce the effect expected if salt bonds are involved in shrinkage.

Similarity of results obtained with various solutions containing inorganic and organic solutes shows common effects of these two types of solute. The results obtained with solutions of organic solutes are the reverse of what might be expected if salt bonds were involved, and therefore it appears that results with electrolytic solutions are also to be ascribed to effects of solute on hydrogen bonds.

The foregoing description is believed to describe successfully the data and observations obtained in these studies.

V. Practical Application

The results of the investigations described in this and in a preceding report [1] appear to have definite practical applications. The method of evaluation of the effect of tannage yields quantitative data of a more fundamental nature than the measurement of shrinkage temperature alone. It has been indicated that organic tanning materials may produce effects comparable to those produced by chrome, and it is not inconceivable that by proper choice of pH, concentration, etc., these materials might produce acceptable substitutes for chromium in tanning.

A second application lies in the investigation of the effects of salts and solutes in general on collagen. The results shown here indicate that magnesium sulfate might be a good preservative for hide, possibly better than sodium chloride. In order to evaluate the effect of solutes, further studies on specimens immersed for extended periods of time would be required. However, the present investigation shows that such studies would be advisable with magnesium sulfate but useless with lithium chloride, etc. This method appears to offer excellent possibilities as a "screening" test.

The entropy and heat values obtained on specimens tested in aqueous solutions may be of practical significance, but, in view of present

knowledge, it appears unwise to interpret these values.

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