Studies of Collagen Tissue Aging: Thermal Shrinkage of Metabolite-Treated Collagenous Tissues.* (27851)

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In the preceding communications(1), attention has been directed to the swelling effects of various intermediary metabolites on hide powder collagen. On the basis of these data, it appears likely that tanning, or cross-linking reactions, in the sense of stabilization of the collagen lattice structure to the adverse effects of either pH or collagenase activity occur between hide powder collagen and only those intermediary metabolites which are aldehydes. The data are conclusive, however, only with respect to the swelling and degradative phenomena of powdered collagen. For this reason, the present study was undertaken to determine whether formed collagenous tissues similarly behave as if tanned after interaction with aldehyde metabolites.

Experimental. Tissue samples of skin, tendon, aorta and bone were obtained from goats and dogs without regard either to age or sex of the animal. The samples of the goat skin were obtained from Amritzar, India† and had been preserved in sun-dried mud and salts. The skins were washed in water for 48 hours, followed by immersion in a 3% sodium sulfide bath for about one-half hour. They were then placed in 25% $Ca(OH)_2$ for 3 days, to pulp and disintegrate the hair and keratin layers, and later washed in water till clear. The epidermal layers were removed by a fleshing machine following which the hides were again washed in water overnight. Finally, they were bated in a 1¼% aqueous solution of Oropom A.S. (Rohm and Haas Co., Philadelphia) at 96-97°F for 2-3 hours, then washed and stored in water in the cold. Immediately prior to use, the hides were cut by hand into 3 × 0.5 inch strips and placed into the metabolite solutions (*vide infra*).

Achilles tendon, aorta and long bone (tibia) specimens were obtained from adult dogs immediately after sacrifice. The specimens were freed of surrounding soft tissues and washed briefly in cold, running tap water. The bone specimens were cut into 3 × 0.1 × 0.05 inch lengths and placed into a solution (pH 7.0) of disodium ethylene-diamine-tetraacetate for 9 days at room temperature. At this time the specimens were quite malleable and, after washing in water, were used without any attempt to quantitate their residual calcium content. Decalcified bone specimens were then handled in exactly the same manner as the other non-mineralized collagenous tissues. Aortae were used as such without removal of the intimal or elastic tissue layers. The sheath of the Achilles tendon was removed and only the tendon itself studied.

All tissues were incubated both at room temperature and at 40°C in freshly prepared 0.15M solutions of various intermediary metabolites of the Embden-Meyerhoff, direct oxidative, and Kreb's cycle pathways‡ (Fig. 1). After stipulated periods of interaction at the temperature levels, each hide preparation was washed in distilled water immediately prior to determination of the shrinkage temperature.

All specimens were suspended vertically in a one liter glass beaker containing distilled water and were attached by appropriately placed metal clips to a Theis-Thomas shrink-

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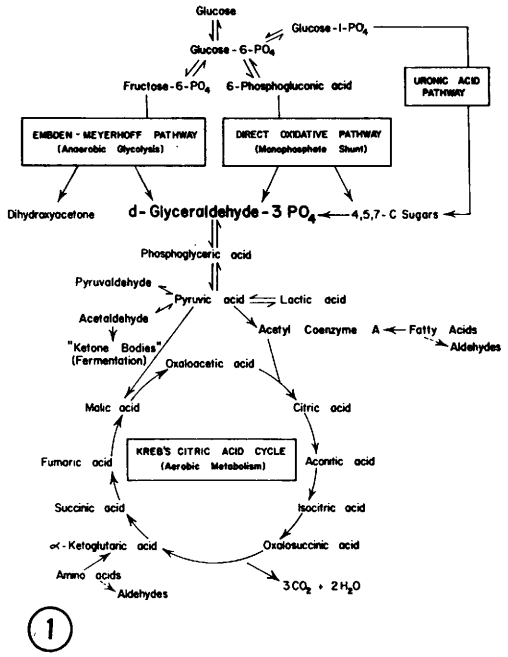
‡ The compounds studied included: glucose, glucose-6-phosphate, fructose-6-phosphate, 6-phosphogluconic acid, dl-glyceraldehyde, phosphoglyceric acid, lactic acid, sodium lactate, pyruvic acid, sodium pyruvate, pyruvaldehyde, oxalacetic acid, citric acid, aconitic acid, α-ketoglutaric acid, succinic acid, sodium succinate, potassium fumarate, malic acid, glycerol, glyoxal, formaldehyde and urea.



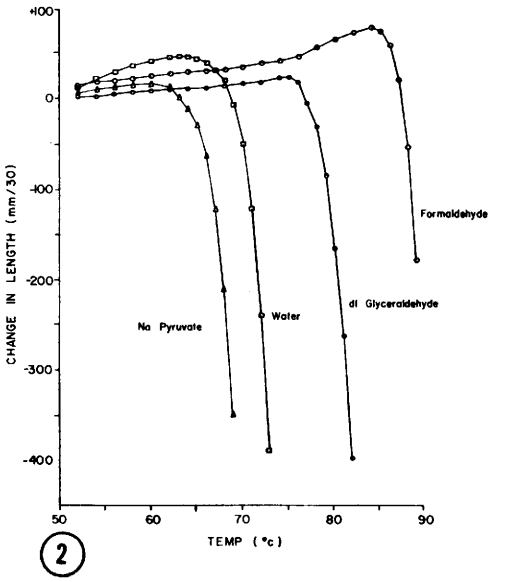


FIG. 1.

FIG. 2. Thermal shrinkage of goat skins in presence of sodium pyruvate, dl-glyceraldehyde and formaldehyde.

age meter. A 165 g counterweight was used in all studies and the shrinkage meter so geared that 1 mm shrinkage in the tissue specimen changed the dial reading by 30 mm. The initial bath temperature was 49°C. Each specimen was equilibrated at this temperature for 30-60 seconds. The temperature was then increased at the rate of 3.7 ± 0.5°C per minute. Dial readings, corresponding to measurements of specimen length, were recorded for all temperatures greater than 50°C and were expressed in terms of change in length with respect to temperature. The instantaneous shrinkage temperature (T_s) was taken as the temperature at which unequivocal shrinkage was first noted. Usually this could be most easily recorded as the range, within 1-2°C, within which shrinkage was apparent.

Results. The shrinkage range of the skin specimens was readily determined after incubation at room temperature or at 40°C either in water or phosphate buffer solutions. The other collagenous tissues disintegrated during treatment in the bath fluids or rapidly shred when placed in the water bath of the shrinkage apparatus. This occurred after treatment at both temperature levels in all metabolite solutions except those of the aldehydes. Hence, no comparable control data could be provided for the collagen materials in these other metabolites. Aldehyde-treated preparations were, on the other hand, quite stable even after incubation at 40°C.

Representative data on the thermal shrinkage of goat skins in certain of the metabolite solutions are presented in Fig. 2 and Tables I and II. It is apparent that, with the exception of formaldehyde, freshly prepared dl-glyceraldehyde solutions produced the most striking and consistent increase in shrinkage temperature. Furthermore, increased resistance of the glyceraldehyde-treated hides to

TABLE I. Thermal Shrinkage of Goat Skins in Presence of 0.15M Aqueous Solutions of Various Solutes. pH_f is the equilibrium pH of the bath fluid. Specimens were incubated for 11 days at 25°C in each of the test solutions.

Solute (0.15M in H_2O)	Bath pH_f	Shrinkage temperature (°C) Range	Change
Water	6.75	64–65	0
Glyoxal	3.50	71–72	(+) 7
Glyceraldehyde	4.90	75–76	(+) 11
Formaldehyde	6.30	84–86	(+)20–21
Na pyruvate	7.35	60–62	(−) 3–4
Na lactate	7.50	60–62	(−) 3–4
Urea	7.80	62–63	(−) 2
Na succinate	8.20	62–63	(−) 2

TABLE II. Thermal Shrinkage of Collagenous Tissues in Presence of 0.15M Glyceraldehyde Solutions in Water and Phosphate Buffers. Tissues were incubated under varying conditions of time and temperature. pH_f is the equilibrium pH after each stipulated interval of time. T_s indicates instantaneous (thermal) shrinkage temperature.

Tissue	Liquid	Time	Temp (°C)	pH_f	T_s (°C)	ΔT_s (°C)
Hide	H_2O	—	25	6.75	64–65	0
	.15M G in H_2O	3 hr	25	6.4	64–65	0
		9 days	15	4.4	70–71	(+) 6
		9 "	25	4.5	75–76	(+) 11
		11 "	15	5.2	70–71	(+) 6
		11 "	25	4.9	75–76	(+) 11
	.15M G in .15M PO_4	2 hr	40	7.35	71–72	(+) 7
		3 "	25	7.4	64–65	0
		2 days	40	7.35	75–76	(+) 11
		8 "	25	6.45	73–74	(+) 9
		8 "	40	5.8	73–74	(+) 9
	.15M G in .30M PO_4	2 hr	40	7.2	73–74	(+) 9
		8 days	25	6.8	73–74	(+) 9
		8 "	40	6.65	73–74	(+) 9
Aorta	.15M PO_4	2 days	40		disintegrated	
	.15M G in .15M PO_4	2 hr	40	7.35	76–77	(+) 12
		2 days	40	7.4	76–77	(+) 12
Tendon	.15M PO_4	2 days	40		disintegrated	
	.15M G in .15M PO_4	2 "	40	7.4	75–76	(+) 11
Decal. bone	.15M PO_4	2 days	40	7.4	64–65	0
	.15M G in .15M PO_4	2 "	40	6.75	82–83	(+) 18

thermal degradation occurred over a remarkably wide range of pH, ionic strength and temperature conditions.

Autoxidation of the dl-glyceraldehyde solutions at 40°C or 60°C for several days (during which interval the solutions became deeply colored and progressively more acidic) was attended by virtually complete loss of tanning properties. Indeed, skin specimens which seemed grossly intact under ambient temperature conditions rapidly disintegrated incubated at 40°C with the solutions of the glyceraldehyde condensation products. Furthermore, the shrinkage temperature of the samples of skin which had been incubated at room temperature was significantly reduced when compared with the specimens treated by the freshly prepared glyceraldehyde solution. Similar observations were made with tendon, aorta and decalcified bone specimens, except that the shrinkage temperature elevation was considerably greater with decalcified bone than the other tissues.

Discussion. The present data not only corroborate earlier findings in the present series, but also are in agreement with previously recorded data on tanning processes in general(2). They also again confirm the occurrence of marked disorganization and impaired stability of hide protein in water and other solutions at temperatures often significantly less than the usual shrinkage temperature of mammalian corium(3-5). This incipient shrinkage presumably differs only in degree from instantaneous shrinkage; both apparent "types" are actually manifestations of a rate process involving the rupture of stabilizing cross-links (hydrogen bonds or other covalent bonds).

Under the currently described experimental conditions, therefore, it would appear that all carbohydrate metabolites other than those with the aldehyde structure result in disruption of the collagen lattice structure. The aldehydes, however, markedly increase the stability of insoluble collagen under identical experimental conditions. This is quite marked in the case of glyceraldehyde, the one major intermediary through which all known carbohydrate metabolic pathways must pass. This stabilization, however, is evidently limited to specific aldehydes(2) and not either to all

aldehydes or under all conditions. As demonstrated here, highly polymerized glyceraldehyde behaves in a manner precisely opposite to that of the monomeric glyceraldehyde.

The chemical details of the apparently involved mechanism will be described later. However, the mechanism appears to involve at least 2 chemical phenomena in a manner quite analogous: (1) to the observed increased fixation of highly aggregated chromium compounds and vegetable tannins by heat-denatured collagen(6,7) due to rupture of hydrogen bridges in adjacent keto imide groups and (2) the intermolecular or interchain cross-linking of globular proteins(8) and polynucleotides by aldehydes(9). Presumably, the first step involves disruption of the stabilizing *intra-molecular* cross links of the molecule (*e.g.*, as in the collagen-gelatin transition) and the second step of an *intermolecular* reaction between the aldehyde group and the protein groups thus freed (2,10).

Summary. The thermal shrinkage of a number of collagenous tissues (skin, aorta, tendon, and decalcified bone) has been studied following incubation with a series of carbohydrate metabolites. The data indicate that only glyceraldehyde among the metabolites studied here, is capable of acting as a tanning or cross-linking agent for collagen under a variety of experimental conditions. These latter roughly approximate the pH, ionic strength and temperature conditions of the extracellular body fluids.

Addendum: Additional details on the relationship between observed tanning properties and the chemical structure of a large number of aldehydes have recently been published (Milch, R. A., *J. Amer. Leather Chems. Assn.*, 1962, v57, 581).

1. Milch, R. A., *Gerontologica,* in press.
2. Gustavson, K. H., (a) *The Chemistry of Tanning Processes*; (b) *The Chemistry and Reactivity of Collagen,* Academic Press, New York, 1956.
3. Weir, C. E., *J. Am. Leather Chem. Assn.*, 1949, v44, 108.
4. Gustavson, K. H., *Nature,* 1960, v188, 419.
5. Rigby, B. J., *Biochem. Biophys. Acta,* 1961, v47, 534.
6. Gustavson, K. H., *Acta Chem. Scand.*, 1947, v1, 581.
7. ———, *J. Am. Leather Chem. Assn.*, 1955, v50, 239.
8. Fraenkel-Conrat, H., Mecham, D. K., *J. Biol. Chem.*, 1949, v177, 477.
9. Haselkorn, R., Doty, P., *ibid.*, 1961, v236, 2738.
10. Walker, J. F., *Formaldehyde,* Am. Chem. Soc., Monograph Series No. 98. Reinhold Publ. Co., New York, 1944.

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Studies of Collagen Tissue Aging: Degradation of Glyceraldehyde-Treated Hide Collagen.* (27852)

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In a previous communication(1) it has been indicated that only those intermediary metabolites which are aldehydes are apparently able to act as tanning agents for hide powder collagen. Tanning in this context has been defined primarily by the relative repression at equivalent equilibrium pH's of the swelling phenomena of metabolite-treated as contrasted to untreated hide powders. As such, one, but only one of the several requisites for tannning ability have been fulfilled (2). In this report are recorded additional data indicating that improved stability of collagen preparations follows interaction with such metabolites.

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