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Studies of Alcaptonuria: Collagenase Degradation of Homogentisic-Tanned Hide Powder Collagen.* (26574)

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Studies in this laboratory on the pathogenesis of alcaptonuric arthritis, the third of the 3 temporally related, characteristic components of the alcaptonuria syndrome (homogentisic aciduria, or alcaptonuria; ochronosis; alcaptonuric, or ochronotic arthritis) have been concerned primarily with the interactions of various homogentisic acid solutions with collagen. Since it had been shown previously(1) that homogentisic acid does not interact with unbound chondroitin sulfate, it followed logically that interactions with collagen, the other and major component of cartilage, as well as the other connective tissues, necessarily was implicated.

Indeed, it was later shown(2,3) that homogentisic acid autoxidation polymers (HGA-OP), in diametric opposition to unoxidized, monomeric homogentisic acid solutions (HGA), become more or less irreversibly bound to collagen by a pH-independent reaction. For steric reasons, this results in significant cross-linking of adjacent collagen polypeptide chains and stabilization of the collagen structural lattice. As such, the resulting tanning reaction would be expected to result in a product which would not only resist water solubilization and heat degradation, as has been previously demonstrated, but would also be expected to result in increased resistance to the action of a number of proteolytic enzyme systems. The present report notes the result of one such study.

Material and methods. Twelve gram batches of limed and neutralized (pH 5.4) bovine hide powder collagen (American Standard Hide Powder, purchased from Frank F. Marshall, Ridgeway, Pa.) were passed through No. 20 ASTM sieves, placed in covered Ehrlenmeyer flasks and reacted for approximately 75 hours at room temperature (21°-28°C) with 500 ml aliquots of water or of aqueous, autoxidized, polymeric homogentisic acid (HGA-OP) solutions (3 mg/ml), prepared as outlined previously(4). The liquid phase was removed by suction filtration after the period of interaction and the solid (collagen) phase was freeze-dried. (A considerable quantity of the previously untreated hide powder collagen was lost by solubilization during water treatment, whereas significantly smaller amounts were solubilized during interaction with HGA-OP. No attempt was made here, however, to provide quantitative data on the precise degree of solubilization in either instance.)

Two hundred milligram samples of each collagen preparation were placed into dry, chemically clean screw-top 15 ml graduated

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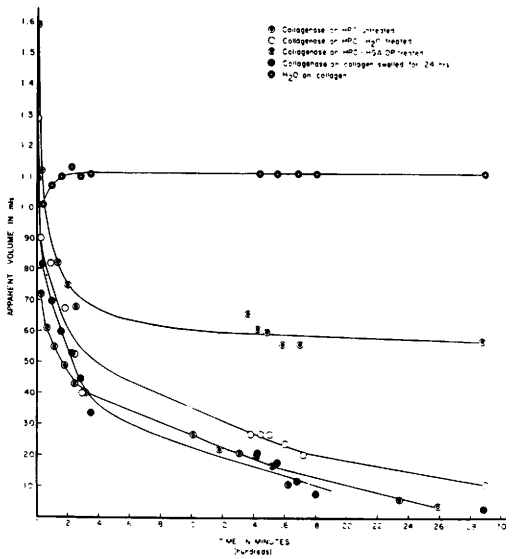

FIG. 1.

centrifuge tubes. The collagen preparations were: (1) untreated, isoelectric hide powder collagen, directly suspended in 10 ml water (pH 6.10); (2) untreated, isoelectric hide powder collagen permitted to swell in 10 ml H_2O for 24 hours before exposure to collagenase; (3) untreated, isoelectric hide powder collagen, directly suspended in the collagenase solutions; (4) isoelectric hide powder collagen pre-treated with water (pH 6.10) as outlined here, then swelled in water for 24 hours before interaction with collagenase, and (5) HGA-OP pre-treated hide powder collagen, prepared as outlined here, then permitted to swell in water at pH 6.10 for 24 hours before exposure to the collagenase solution.

After treatment as outlined immediately above, 10 ml of a collagenase solution (4 mg/ml, lot No. 5732, purchased from Worthington Biochemical Corp., Freehold, N. J.) were then layered on to certain of the collagen preparations and thoroughly stirred to effect dispersion of the solid phase. At appropriate time intervals, the tubes were centrifuged at 2500 rpm at room temperature for 5 minutes. The mean apparent hydrated volume of the solid phase (minimum of 5 tubes) was read to the nearest 0.1 ml and estimated to the nearest 0.05 ml from the graduations on the side of each tube. The tubes were then restirred and permitted to stand in air until the next determination was made.

Initial pH (pH_i) and final pH (pH_f) values of the enzyme solutions were determined with a Beckman Zeromatic pH meter (instrumental error, 0.03 pH).

Results. The mean apparent hydrated volume of the solid (collagen) phase in presence of water and collagenase solutions is plotted against time of interaction in Fig. 1 and summarized in Table I.

Discussion. A number of investigators have suggested certain technical problems, reviewed in detail by Robb-Smith(5) and Gus-

TABLE I. Summary of Observations.

Collagen phase	Liquid phase	Treatment	pH_i	pH_f	ΔpH	Mean app. vol (ml at 1400-1800 min.)	% $\wedge$
Untreated H.P.C.	H_2O	Direct. susp.	6.10	5.00	−1.10	1.11	—
Idem	Collagenase	*Idem*	6.07	6.22	+ .15	.14	87.4
"	"	H_2O-swelled	6.05	6.65	+ .60	.15	86.5
H_2O-treated H.P.C.	"	*Idem*	5.90	6.25	+ .35	.24	78.4
HGA-OP-treated H.P.C.	"	"	5.90	5.48	− .42	.60	45 9

"H.P.C." signified limed and neutralized (isoelectric) bovine hide powder collagen. "Direct. susp." indicates that hide powder collagen preparation was directly suspended in liquid phase. "H_2O-swelled" indicates that collagen preparation was permitted to swell in water (pH 6.01) for 24 hr before water was decanted and collagenase solution added. pH_i is initial and pH_f final pH of liquid phase. "Mean app. vol" = mean apparent hydrated vol, used here as a convenient and rough estimate of relative effects of water and collagenase on various hide powder collagen preparations, and arbitrarily taken (mean value of all apparent volume determinations) between 1400 and 1800 min. after beginning of interaction with collagenase. "Percent change" is presented in terms of calculated percentage change in mean apparent hydrated volume of collagenase-treated preparations relative to that of untreated hide powder preparation directly suspended in water.

tavson(6,7), to indicate that mammalian hide powder collagen, in contradistinction to native collagen, is not a suitable agent for investigation of proteinase action. This is largely attributable to the facts that hide powder contains a mixture of both native and heat denatured collagen, that "limed skin tissue(pelt) used in the isoelectric range is too resistant," and, in general, that any pretreatment of native collagen necessarily imparts a modification of molecular structure and organization, and hence of the properties of native collagen.

For the present basically qualitative purposes, however, a precise knowledge of the chemical nature of either starting material or split-products would not be necessarily critical. If a tanning phenomenon does occur when limed and neutralized hide powder collagen is interacted with autoxidized, polymeric homogentisic acid (HGA-OP) solutions, then by definition, it is required only to demonstrate that the pre-treated product (HGA-OP bound collagen) possesses a significantly increased resistance to collagenase in comparison to that of the untreated product. This is precisely what was observed.

Analysis of the kinetic data clearly indicates that pH *per se* is not a factor in the observed changes since there was a distinct difference in the effects of collagenase solutions and water controls at exactly the same initial pH. Furthermore, since only a negligible and probably insignificant final effect could be attributed to swelling of the isoelectric collagens in water before addition of collagenase, it follows that this experimental procedure is likewise not determining in the present observations. It appears, therefore, that the measured differences in rate, magnitude and calculable percentage change in apparent hydrated volume of the untreated and HGA-OP treated collagens in the presence of collagenase are highly significant.

Thus, in expansion of earlier data and in support of additional (unpublished) data indicating decreased chemical reactivity of collagen in consequence of treatment with HGA-OP, it appears that HGA-OP acts in all classical manners as a potent tanning agent for hide powder collagen.

Summary. A volumetric study has been conducted of the effects of collagenase solutions on isoelectric and treated bovine hide powder collagen preparations. The data indicate that the enzyme solution used is active against hide powder collagen, that the effects of the collagenase noted here are independent of pH and that pretreatment of hide powder with the autoxidation polymers of homogentisic acid (HGA-OP) results in significant diminution of the effects of collagenase. With earlier data indicating that HGA-OP binds to hide powder, the current data are interpreted as indicating that HGA-OP acts as an effective tanning agent for hide powder collagen.

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