Studies of Alcaptonuria: Binding of Homogentisic Acid Solutions to Hide Powder Collagen.* (26241)

ROBERT AUSTIN MILCH
(With the technical assistance of Richard A. Murray)
Division of Orthopaedic Surgery, The Johns Hopkins University School of Medicine and Johns Hopkins Hospital, Baltimore, Md.

Data have been presented previously to indicate that unoxidized homogentisic acid solutions can interact with hide powder collagen preparations(1). It has also been shown (2) that the interaction with unoxidized homogentisic acid (HGA) is largely a reversible phenomenon, whereas interaction with polymerized, autoxidized homogentisic acid solutions (HGA-OP) presumably involves actual binding to collagen. The present studies were undertaken in an attempt to provide quantitative data on these interactions.

Materials and methods. One hundred milligram samples of commercially available limed hide powder collagen (American Standard Hide Powder†), neutralized to the isoelectric point (pH 5.4) were passed through No. 20 ASTM sieves and placed in chemically clean, dry cellulose ultracentrifuge tubes.‡ Ten ml of each of the test solutions (*vide infra*) were then layered on to the collagen preparations and thoroughly stirred to effect dispersion of the solid phase. The tubes were permitted to stand in air at room temperature (25° C) for 24 hours and from 1 to 2 weeks, with occasional stirring. At stipulated intervals (Table I), tubes were centrifuged in a Spinco analytical ultracentrifuge at 30,000 rpm (59,310 × g) for 5 minutes. The particle-free supernatants were decanted and their absorbance determined using a Beckman DU spectrophotometer with photomultiplier attachment.

Similar studies were performed using acetylated hide powder collagen and Lyon poudre de peau blanche (Inst. de Recherches pour les Industries du Cuir, Lyon, France).§ The acetylated collagen was prepared by reacting isoelectric American Standard Hide powder collagen with acetic anhydride in fully saturated sodium acetate as described previously(1,3,4).

The test solutions were D_2O,¶ freshly prepared 0.03 M HGA, and HGA-OP prepared by autoxidation of 0.03 M HGA solutions. All solutions were employed in a concentration range such that physical or optical density of each adhered to the Beer-Lambert law.

Physical density of D_2O solutions was determined by the falling-drop method(5) using xylene: bromobenzene mixtures (79:21) for the liquid phase and a specially designed, calibrated microliter syringe assembly which gave reproducible readings to the fifth decimal place. Optical density of HGA solutions was determined from absorbance observations at the absorption maximum at 2900 Å or along the linear slope of the absorption peak at 3250 Å. HGA-OP solutions were read at 3750 Å.

Binding was determined by the percentage change in density of the supernatant liquid phase before and after the period of interaction with each adsorbent by:

$$\%\ \text{``bound''} = \%\ \text{bound/100 mg adsorbent} = \frac{100\ (D_o - D_f)}{D_o}$$

where, D_o = initial density (optical or physical) of liquid phase, and D_f = final density, after interaction with the adsorbent.

Initial (pH_i) and final (pH_f) pH values of the supernatant liquid phase were determined using a Beckman zeromatic pH meter

* Aided by a grant from Nat. Inst. of Arthritis and Metab. Dis., N.I.H., U. S. Dept. of H.E.W.

† Purchased from Frank F. Marshall, Ridgeway, Pa.

‡ No. 2235, 5/8 × 3 ins., purchased from Spinco Beckman, Palo Alto, Calif.

§ Kindly supplied by Prof. K. H. Gustavson, Swedish Tanning Research Inst., Stockholm.

¶ Purchased from Volk Radiochemical Co., Chicago, Ill.

TABLE I. Extent of Binding of Unoxidized, Monomeric Homogentisic Acid (HGA) and Autoxidized, Polymeric Homogentisic Acid (HGA-OP) Solutions to American Standard Hide Powder Collagen (HPC). Acetylated hide powder collagen indicated by AcHPC and deuterium oxide by D_2O.

Reactants	Time	pH_i	pH_f	Δ pH	% bound
HGA + HPC	24 hr	3.32	3.35	+ .21	3.0
D_2O + HPC	"	3.52	4.65	+1.13	.01
HGA-OP + HPC	"	3.37	3.57	+ .20	69.3
HGA-OP + AcHPC	"	3.36	3.47	+ .11	66.2
HGA-OP + HPC	1 wk	3.33	3.85	+ .52	68.0
HGA-OP + HPC	2 "	3.35	4.62	+1.27	62.2
HGA-OP + AcHPC	2 "	3.35	3.85	+ .50	62.1

pH_i of all solutions was not adjusted except for that of D_2O. This was adjusted from pH 5.4 to pH 3.5 by addition of HCl (0.1N).

(instrumental error 0.03 pH).

Results. The extent of binding of each of the test solutions to American Standard hide powder collagen preparations at pH approximately 3 is given in Table I. Results with Lyon poudre de peau blanche were essentially identical and are not included here.

Under the described experimental conditions, it is quite clear that: (1) neither D_2O nor HGA bind to hide powder collagen to any appreciable extent; (2) there is significant binding of HGA-OP solutions to hide powder collagen; (3) HGA-OP binding is unaffected by acetylation of the collagen amino acids, and (4) HGA-OP binding is essentially unaffected by the time of interaction with the hide powder collagen preparations.

Discussion. These observations on the extent of binding of the various homogentisic acid solutions by hide powder collagen corroborate previous qualitative data on collagen swelling effects of homogentisic acid solutions(1) and semi-quantitative observations on adsorption of homogentisic acid solutions on to collagen chromatographic columns(2).

Furthermore, these data provide confirmation of the earlier inference that although monomeric, unoxidized homogentisic acid interacts with hide powder collagen in the pH range below that of the isoelectric point of the collagen preparation, this particular interaction is markedly pH-dependent and does not in fact involve a binding mechanism. Since it has been shown previously that there is no appreciable interaction between these 2 moieties at pH's at or above the isoelectric point, it would appear unlikely that monomeric homogentisic acid would have much if any effect on collagen *in vivo*.

With autoxidation of the colorless monomer to the pigmented oxidation polymer(s), however, there is a distinct and unequivocal difference in the observed interactions with collagen. Pronounced binding of polymerized homogentisic acid solutions (HGA-OP) to the collagen occurs quite irrespective of the pH of the HGA-OP solutions. In these instances, observed swelling effects are more or less irreversible, even after removal of the HGA-OP solution, are entirely independent of pH and are associated with little or no solubilization of collagen(1). Indeed, HGA-OP has all characteristics, including chemical binding as noted here, of a potent tanning agent for hide powder collagen. This tanning property, which is entirely lacking in the unoxidized monomeric solutions (HGA) is present not only at pH values, but also at ionic strengths which are characteristic of body fluids.

It is not unreasonable to suggest, therefore, that this tanning phenomenon may account for the morphological sequellae observed in alcaptonuria. In such a view, ochronosis and arthritis in patients with homogentisic acid retention (alcaptonuria) could arise in consequence of some mechanism (other than tyrosinase catalysis(6)) which brings about polymerization of HGA to HGA-OP, which, when once formed, then could chemically bind to collagen and result in collagen tannage.

Since acetylation of the collagen amino acid groups did not appreciably alter the ex-

tent of HGA-OP binding, the chemical bond formed between the two moieties presumably would be primarily of the non-ionic, coordinate (hydrogen bond and van der Waal's) type. For steric reasons, the tanning agent (HGA-OP) would probably act as a multifunctional agent to form such primarily coordinate inter-chain cross-links between adjacent collagen polypeptide chains(2). The net effect would be stabilization and "hardening" of the collagen lattice structure.

Summary. Binding of monomeric, unoxidized (HGA) and polymeric, autoxidized (HGA-OP) homogentisic acid solutions to hide powder collagen preparations has been studied. It has been shown that there is no active binding of HGA to collagen, whereas there is extensive binding of HGA-OP to collagen. Coupled with previously presented observations, it is suggested that HGA-OP can act as a potent tanning agent for collagen. It is further suggested that ochronosis and degenerative joint disease observed in patients with alcaptonuria may possibly be explained by the *in vivo* occurrence of such a phenomenon.

1. Milch, R. A., *Arthritis and Rheumatism,* 1960, in press.

2. Milch, R. A., Murray, R. A., *ibid.,* 1960, in press.

3. Fraenkel-Conrat, H., in *Amino Acids and Proteins,* D. M. Greenberg, ed., C. C Thomas, Springfield, Ill., 1951, pp. 532-585.

4. Gustavson, K. H., personal communication.

5. Barbour, H. G., Hamilton, W. F., *J. Biol. Chem.,* 1926, v69, 625.

6. Milch, R. A., Schirmer, H. K. A., *Arch. Biochem. Biophys.,* 1960, v89, 27.

Received October 20, 1960. P.S.E.B.M., 1961, v106.

Anaphylaxis in *Bordetella pertussis*-Treated Mice. IV. Schultz-Dale Reaction.* (26242)

J. MUNOZ AND MAUNG MAUNG

Stella Duncan Memorial Research Laboratories and Department of Microbiology, Montana State University, Missoula

Injection of *Bordetella pertussis* cells into mice increases the susceptibility of these animals to fatal anaphylactic shock, whether the shock is induced by passive or active means(1). *B. pertussis* cells also increase susceptibility of mice to various agents and stresses such as histamine, serotonin, cold stress, x-rays, etc.(1). *B. pertussis* does not, however, increase susceptibility of the mouse skin to passive cutaneous anaphylaxis (PCA) (2). The mechanism by which *B. pertussis* produces the changes observed is not well understood, although *B. pertussis* may indirectly interfere with adrenal function(3). *B. pertussis* enhances antibody response to various antigens(4,5), and most likely increases the susceptibility of mice to active anaphylaxis by stimulating antibody response to sensitizing antigen. In spite of the adjuvant activity of *B. pertussis,* the relationship of this effect to the increased susceptibility to anaphylaxis produced in mice by these organisms has been questioned(4,5). Irrespective of the role that the adjuvant effect has on active sensitization, the increased susceptibility to passively induced anaphylaxis cannot be explained by the adjuvant effect of *B. pertussis,* since in this case it can be shown that *B. pertussis*-treated mice become highly sensitive to fatal anaphylaxis with amounts of antibody that do not sensitize normal mice to fatal shock(3,6). In this paper the effect of *B. pertussis* cells on specific active and passive sensitization of smooth muscle will be described.

* This work was supported in part by research grant from Nat. Inst. of Allergy and Infect. Dis., N.I.H., P.H.S.

Materials and methods. Mice. Female mice of the CFW strain weighing from 14 to 18 g purchased from Carworth Farms, New