Evidence for Glycosylated Crosslinks in Body-Wall Collagen of the Sea Cucumber, *Thyone briareus* (37600)

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Reducible interchain crosslinks have been detected in the majority of collagens that have been investigated, including several collagens of invertebrate tissues (1, 2). The invertebrate collagens apparently all contain dehydro-hydroxylysinohydroxynorleucine (dehydro-HylOHNle) as one of their major reducible crosslinks, similar to the situation in the mineralized collagens of bone and dentine and the collagens of cartilage in vertebrates. Collagen in the body-wall of the sea cucumber, *Thyone briareus,* gives a remarkably simple profile of ^{3}H-activity on analysis after reduction with [^{3}H]-$NaBH_4$ and hydrolysis in acid (1). Thus, HylOHNle was identified as the only significant peak of ^{3}H-activity in the elution profile from the amino acid analyzer.

It has been suggested that HylOHNle may be a natural crosslink synthesized by reduction of dehydro-HylOHNle *in vivo* in the collagens of mammalian bone and dentine (3). Recent analyses in our laboratory failed to detect the naturally reduced crosslink, but did indicate that in chicken and bovine bone collagen some of the crosslinks, dehydro-HylOHNle, was present in a more stable, keto-amine rearranged form and was also glycosylated (4). The present report describes the analysis of sea cucumber collagen for possible glycosides of dehydro-HylOHNle, and for HylOHNle derived by natural reduction.

Materials and Methods. The body-wall of *T. briareus* was prepared and reacted with [^{3}H]-$NaBH_4$ (10 Ci/mole) as previously described (1). Samples of this material were hydrolysed in 2 *M* KOH in alkali-resistant glass tubes for 24 hr at 105°. The hydrolysate was neutralized with $HClO_4$ at 4°; the precipitate of $KClO_4$ was removed by filtration, and the filtrate was evaporated to dryness at 40° under reduced pressure. Tritiated compounds in the hydrolysate were fractionated on a single-column, amino acid analyzer by elution with a complex gradient (1). Using a split-stream device half of the column effluent was mixed with scintillation fluid (Aquasol, New England Nuclear Corp., Boston, MA), and monitored for ^{3}H-activity while flowing through a coil in a specially constructed well of an Intertechnique Model SL20 liquid scintillation counter (5).

Results and Discussion, After hydrolysis in 2 *M* KOH, essentially two tritiated compounds were recovered from the reduced *Thyone* collagen, HylOHNle and material in a much larger peak of ^{3}H-activity that eluted near tyrosine (Fig. 1). Treatment of this base hydrolysate with 0.1 *M* HCl at 100° for 24 hr converted the labeled compound that eluted near tyrosine, to a compound that eluted as a peak of ^{3}H-activity immediately before NH_3 (Fig. 1). Such conditions of acid hydrolysis selectively remove the terminal glucose residue from glucosylgalactosylhydroxylysine isolated from a base hydrolysate of collagen (6). The compound that chromatographed near tyrosine was isolated from a base hydrolysate of about 200 mg of the *Thyone* collagen. It gave a single Ninhydrin-positive peak on the analyzer, and after hydrolysis in 2 *M* HCl for 4 hr at 105°, conditions which release most of the glucose and galactose that is bound to hydroxylysine in glucosylgalactosylhydroxylysine (6), only

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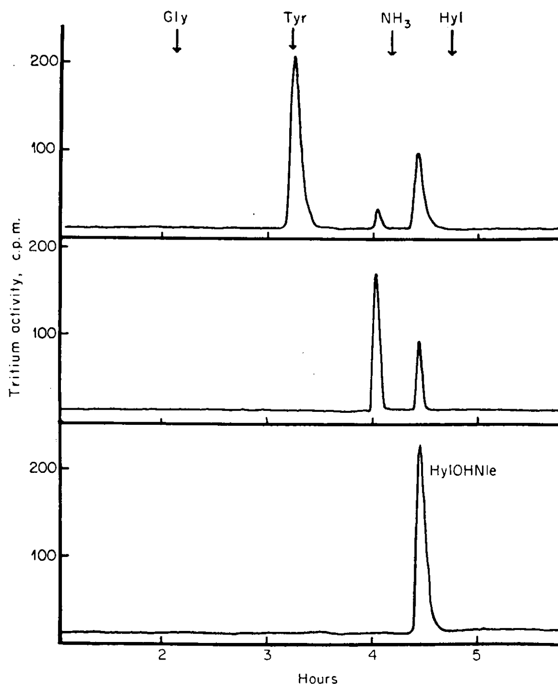

FIG. 1. Chromatograms of tritiated compounds in hydrolysates of [^{3}H]-$NaBH_4$-treated collagen of the body-wall of a sea cucumber, *Thyone briareus*. Upper—collagen hydrolyzed in 2 *M* KOH for 24 hr at 105°. Middle—collagen hydrolyzed in 2 *M* KOH and treated with 0.1 *M* HCl for 24 hr at 105°. Lower—collagen hydrolyzed in 3 *M* HCl for 48 hr at 105°.

HylOHNle and a small amount of the material eluting as a peak immediately before NH_3 were detected. Furthermore, analysis of such a hydrolysate for neutral sugars by thin-layer chromatography on cellulose in *n*-butanol–pyridine–water (6:4:3), and staining with silver nitrate reagent, revealed about equal amounts of glucose and galactose.

These findings can be interpreted if most of the dehydro-HylOHNle crosslinks in body-wall collagen of *Thyone* are glycosylated, *i.e.*, derived from glucosylgalactosylhydroxylysine residues that are known to be present in collagen of this tissue (7). The compound in the peak of ^{3}H-activity eluting near tyrosine appears to be glucosylgalactosyl-HylOHNle, and the compound in the peak eluting before NH_3 is probably galactosyl-HylOHNle, derived from the former compound on mild hydrolysis in acid. It is uncertain at present whether one or both of the two hydroxyl groups in the HylOHNle of the *Thyone* collagen are glycosylated; though it might be expected from the recent results of analysis of a peptide with a glycosylated crosslink from bone collagen that only one hydroxyl is glycosylated (4).

A crosslinked peptide was isolated from calf bone collagen after reduction with $NaBH_4$ and digestion with bacterial collagenase (4). The crosslink in this peptide was HylOHNle and it also appeared to be glycosylated through one of its two hydroxyl groups. The second hydroxyl group in the crosslink apparently had derived by $NaBH_4$-reduction of the keto group of the keto-amine rearranged form of dehydro-HylOHNle. Furthermore, after base hydrolysis of this peptide from bone collagen the crosslinking compound was recovered on the amino acid analyzer mainly as a peak eluting in a similar position to the peak found immediately before NH_3 in the present study, but some was recovered as a peak with an identical elution position near tyrosine to the crosslinking compound from a base hydrolysate of the *Thyone* collagen. The results suggest that glycosylated hydroxylysine residues participate directly in the

biosynthesis of reducible crosslinks in collagen. Such residues could control the location of intermolecular crosslinks in collagen molecules, or perhaps the glycosylated crosslinks are particularly stable crosslinking bonds.

The possibility that dehydro-HylOHNle, either glycosylated or not glycosylated, may be naturally reduced to a more stable crosslink in the *Thyone* collagen, as suggested for the collagens of mammalian bone and dentine (3), was investigated. Large samples (15 to 20 mg dry wt) of *Thyone* body wall were hydrolyzed in 6 *M* HCl for 24 hr at 105°, and eluted from the 60 cm column of the amino acid analyzer with a single buffer, 0.35 *M* sodium citrate, pH 5.25. The amount of HylOHNle in the collagen could be measured directly as a Ninhydrin-positive peak. Thus, the tissue revealed a relatively large peak of HylOHNle after treatment with $NaBH_4$, but none of this compound was detected in the same weight of untreated tissue (Fig. 2).

It is concluded that natural reduction of the crosslink, dehydro-HylOHNle to HylOHNle, is not a significant pathway for crosslink stabilization in body-wall collagen of *T. briareus*. Direct analyses of mammalian and avian bone collagens at various stages of maturity also failed to detect a significant amount of naturally reduced HylOHNle (5, 8). In a previous study neither dehydrohydroxylysinonorleucine nor dehydrolysinonorleucine, the major aldimine crosslinks of mammalian, soft-tissue collagens, could be detected as the naturally reduced crosslinks (9). Thus, collagen fibrils may be effectively stabilized by the reducible crosslinking bonds, particularly those aldimines that are derived from aldehydes of hydroxylysine, which can apparently stabilize by rearrangement from aldimine to keto-amine structure, as suggested by R. Fairweather (4, 10).

Summary. Only one tritiated compound, the reduced crosslink hydroxylysinohydroxynorleucine, is detected by ion-exchange chromatography after $[^3H]$-$NaBH_4$ reduction and acid hydrolysis of body-wall collagen from the sea cucumber, *Thyone briareus*. However, after hydrolysis of the $[^3H]$-$NaBH_4$-treated collagen in 2 *M* KOH rather than in acid, much less of this compound is detected. Instead, a new main peak of ^3H-activity is eluted earlier, near tyrosine.

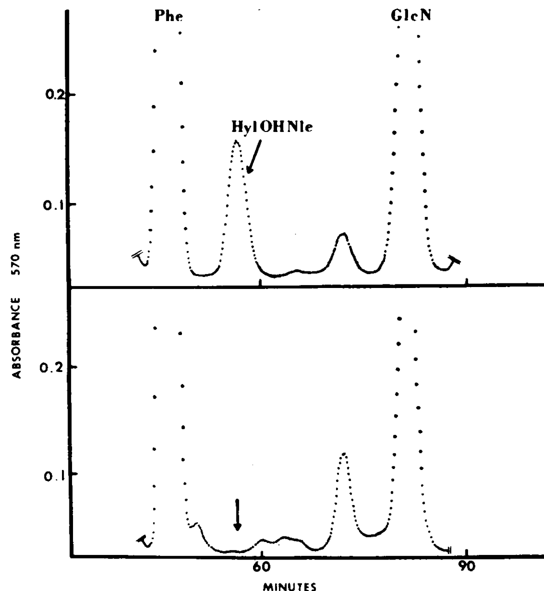

FIG. 2. Chromatographic analysis of the crosslink, HylOHNle, in body wall collagen of sea cucumber hydrolyzed in 6 *M* HCl for 24 hr at 105°. Upper—$NaBH_4$-treated tissue. Lower—untreated tissue.

The compound in this new peak could be converted to hydroxylysinohydroxynorleucine by hydrolysis in 2 *M* HCl, or to an intermediate compound by hydrolysis in 0.1 *M* HCl. About equal amounts of glucose and galactose were detected by thin-layer chromatography after hydrolysis of the isolated compound with the higher concentration of acid. It is concluded that in this invertebrate collagen most of the crosslink, dehydro-hydroxylysinohydroxynorleucine, is glycosylated. Since the reduced form of the crosslink could not be detected by reaction with Ninhydrin in large samples of the collagen, reduction of dehydro-hydroxylysinohydroxynorleucine to hydroxylysinohydroxynorleucine *in vivo* does not appear to be a significant pathway for the maturation of crosslinks in body-wall collagen of *Thyone briareus*.

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