ATTACHMENT OF CARBOHYDRATE TO COLLAGEN. ISOLATION, PURIFICATION AND PROPERTIES OF THE GLUCOSYL TRANSFERASE *

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Collagen, from various sources and in various forms, has carbohydrate associated with the peptide chain (Grassmann and Schleich, 1935; Gross et al., 1958; Kuhn et al., 1959; Blumenfeld et al., 1963). In citrate-soluble collagen from guinea pig skin the carbohydrate has been shown to consist of a disaccharide of glucose and galactose linked O-glycosidically to the hydroxyl group of lysine (Butler and Cunningham, 1965, 1966). Glucosylgalactosylhydroxylysine moieties have also been isolated from tendon collagen and calf skin tropocollagen (Spiro, 1967), and from normal human urine (Cunningham et al., 1967). The present report deals with the isolation from embryonic guinea pig skin and cartilage of the enzyme responsible for transferring glucose-^{14}C from UDP-glucose-^{14}C(UL) to galactose in a receptor prepared from collagen.

Citrate-soluble collagen was prepared by the method of Gallop and Seifter (1963) from the skins of male guinea pigs weighing 350-500 gms. This material contained 0.48% hexose as measured by the orcinol (Winzler, 1955) or anthrone (Seifter et al., 1950) procedures. The most efficient method for releasing the glucose from the collagen involved hydrolysis of the collagen for 30 minutes at 100° C with 1N HCl. The hydrolysate was

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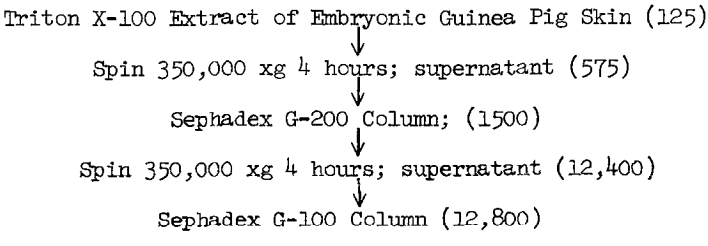
neutralized with 1N NaOH and dialyzed for 16 hours against deionized water. The product, designated as "receptor" in this report, was lyophilized and stored at 0°C over $CaCl_2$. Commercial preparations of calf skin collagen (Calbiochem) and tendon collagen (Sigma) gave results similar to those for the prepared guinea pig skin collagen, which was utilized in all experiments reported below.

Approximately mid-term (30 days) embryonic guinea pigs were removed by section and the skin and cartilaginous ends of the limb bone rudiments were dissected and placed at the temperature of melting ice. Two methods were used to extract the enzyme. In the first method the tissues were homogenized separately in a Virtis homogenizer in cold 0.1M KCl and extracted at 5°C for 16 hours in the same solution. The suspension was passed through several thicknesses of cheese cloth, and the supernatant solution centrifuged at 20,000 xg for 1 hour at 3°C. The resultant pellet was extracted for 16 hours at 5°C with 20 volumes of 0.1% Triton X-100. This suspension was centrifuged at 20,000 xg for 1 hour at 3°C and the supernatant was used as crude enzyme. The second method consisted of simply homogenizing the tissue in 0.1% Triton X-100 and then extracting for 16 hours in 20 volumes of the same solution at 5°C. This suspension was centrifuged at 20,000 xg for 1 hour at 3°C and the supernatant solution employed as the crude enzyme preparation. Crude enzyme prepared by

Figure 1

Purification Scheme
(Number in parentheses refer to cpm/mg protein)



the second method from embryonic guinea pig skin was purified more than 100 fold by the procedures outlined in Figure 1. Gel electrophoresis of this purified enzyme at pH 8.6 according to the method of Reisfeld et al. (1962) showed only one protein band. Purified enzyme was prepared only from the embryonic guinea pig skin extract, although extracts prepared by either method from embryonic guinea pig cartilage contained active enzyme.

The purified enzyme had an optimum temperature of 37°C, optimum pH of 5.8, and was activated by any of four divalent cations. Mn++ was most efficient, followed by Co++, Mg++, and Ca++. The complete assay mixture which was incubated for 45 minutes at 37°C contained 50 µl of the purified enzyme mixture (approximately 715 µg protein), 50 µl of the receptor (approximately 800 µg protein), 10 µl of 0.25M $MnCl_2$, 10 µl of UDP-glucose-^{14}C (approximately 40,000 cpm; 1.4×10^{-10} mole) and 10 µl of 0.1% Triton X-100, to a final volume of 0.130 ml. After 45 minutes incubation, the protein of the reaction mixture was precipitated with 0.1% phosphotungstic acid in 0.5N HCl, and the radioactivity was determined as given elsewhere (Cook et al., 1965). The results of the assay and experiments performed to demonstrate the specificity of the reaction, utilizing a variety of prepared receptors, enzyme preparations and labelled dinucleotide sugars, are shown in Table 1.

To determine the site of attachment of the labelled glucose by this enzyme 12 samples were incubated, precipitated, and washed in the manner described above, except that 250 µl of receptor were used instead of 50 µl. The precipitates were pooled, combined with 100 mg of guinea pig skin collagen, and hydrolyzed in 3.2 ml of 3N NaOH under nitrogen for 6 hours at 105°C. After neutralizing with HCl, the mixture was placed on a P-2 polyacrylamide gel column and eluted with 0.1M acetic acid. Paper electrophoresis at pH 4.7 of the resultant radioactive protein peak revealed 8-10 peptides, only one of which was radioactive. This mixture was fractionated on

Table 1

UDP-glucose-^{14}C Transferred to Receptor
(Assay mixtures were incubated 45 minutes at 37° C)

		CPM
I.	Complete System*	9781
	-$MnCl_2$	2259
	+EDTA (7.5×10^{-7} mole)	154
II.	Complete (-Receptor)	44
	+Native guinea pig skin collagen	71
	+Fetuin	20
	+α_1-Glycoprotein (-sialic acid)‡	60
	+Porcine submaxillary glycoprotein (-sialic acid, fucose)	0
	+Fetuin (-sialic acid)	40
	+Fetuin (-sialic acid, galactose)	60
III.	Complete (-Enzyme)	134
	+80° C Treated enzyme	106
	+Embryonic guinea pig cartilage extracted by Method 1 (approximately 980 µg protein)	4265
IV.	Complete (-UDP-glucose-^{14}C)	0
	+UDP-galactose-^{14}C	97
	+UDP-N-acetylgalactosamine-^{14}C	287
	+GDP-mannose-^{14}C	190

*Complete system contained: 50 µl purified embryonic guinea pig skin enzyme preparation, 50 µl receptor preparation, 10 µl of UDP-glucose-^{14}C, 10 µl of 0.1% Triton X-100, and 10 µl of 0.25M $MnCl_2$. Receptor materials were tested at the same concentration as the collagen receptor. The dinucleotide sugars were tested at the same level of cpm as the UDP-glucose-^{14}C.

‡α_1-glycoprotein (-sialic acid) refers to α_1-glycoprotein in which the sialic acid has been removed by acid or enzymatic degradation leaving an exposed galactose residue; similarly for the other receptors.

Dowex-50, by eluting with a pH gradient (Schroeder et al., 1962), into 8 distinct peptide fractions, 1 being radioactive. This fraction, which contained only one peptide on electrophoresis at pH 4.7, was hydrolyzed in either 6N or 1N HCl at 105° C for 11 hours. High voltage electrophoresis at pH 4.7 and chromatography in two systems--pyridine:water:ethyl

acetate:acetic acid (5:3:5:1) and butanol:acetic acid:water (4:1:5)--indicated the radioactive fraction contained only hydroxylysine, glucose and galactose, the radioactivity residing entirely with the glucose. Amino acid analysis on the amino acid analyzer (Beckman) showed only one amino acid: hydroxylysine. Gas chromatography of the alditols, prepared and analyzed by the method of Kim (1967), revealed only galactose and glucose. Quantitation of the combined data gave a molar ratio (based on hydroxylysine as 1) of 1.0 : 0.89 : 0.92 (hydroxylysine:galactose:glucose). It can be concluded, therefore, that the glucosyl-galactose disaccharide is being synthesized by this enzyme.

In addition to testing the glycoprotein receptors with available galactose residues listed in Table 1, galactose, galactosamine, and N-acetylgalactosamine were substituted for the collagen receptor in the assay procedure to see if a disaccharide might be formed. Each reaction mixture was tested chromatographically in isobutyric acid:ammonium hydroxide:water (57:4:39) for 16 hours. No evidence of a disaccharide was found; in each instance all the radioactivity continued to be associated with UDP-glucose or glucose 1-phosphate.

Glucose is rarely found as a component of glycoproteins (Eylar, 1965). The results presented in this report indicate that the enzyme responsible for the synthesis of the glucose-galactose bond in the collagen molecule is highly specific, and apparently its action is dependent on hydroxylysine or the amino acid sequence about the hydroxylysine to which the galactose is attached. None of the other receptors such as desialyzed α_1-glycoprotein, fetuin, or PSM were functional. This high degree of specificity may be necessary to distinguish between collagen molecules and other glycoproteins. The hypothesis is suggested that if the carbohydrate of secreted glycoproteins acts as a marker or label for exportable proteins (Eylar, 1965) the attachment of galactose and glucose to hydroxylysine in

collagen could conceivably function as part of the control mechanism signaling completion of the molecule and initiating its release. Since both proline and lysine are hydroxylated after they have been incorporated into long polypeptide precursors of collagen by protocollagen hydroxylase (Kivirikko and Prockop, 1967), and after the complete protocollagen polypeptides are released from the ribosomes (Bhatnagar et al., 1967), the addition of the hexoses is most probably a post-ribosomal event.

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