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Characterization of the Oligosaccharides of Prolyl Hydroxylase, a Microsomal Glycoprotein†

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ABSTRACT: Prolyl hydroxylase is a tetrameric glycoprotein that catalyzes a vital posttranslational modification in the biosynthesis of collagen. The enzyme purified from whole chick embryos (WCE) possesses two nonidentical subunits, α and β, and has been shown by several techniques to reside in the endoplasmic reticulum of chick embryo fibroblasts. The studies described here demonstrate that the larger of the two subunits (α) exists in two forms in chick embryo fibroblasts (CEF); these two forms differ in carbohydrate content. The larger α subunit, α', contains two N-linked high mannose oligosaccharides, each containing eight mannose units; the smaller subunit, α, contains a single seven-mannose N-linked oligosaccharide. Both oligosaccharides could be cleaved by endo-β-*N*-acetylglucosaminidase H and completely digested with α-mannosidase to yield mannosyl-*N*-acetylglucosamine.

Prolyl hydroxylase [prolyl-glycyl-peptide, 2-oxoglutarate: dioxygenase (4-hydroxylating), EC 1.14.11.2] catalyzes a critical posttranslational modification during the biosynthesis of collagen (Kivirikko & Myllyla, 1980; Davidson & Berg, 1981). Electron microscopy with ferritin-labeled antibodies to prolyl hydroxylase and subcellular fractionation have demonstrated that the enzyme is located in the endoplasmic reticulum of fibroblasts (Olsen et al., 1973; Peterkofsky & Assad, 1976). It has been purified from a number of sources (Kivirikko & Myllyla, 1980) and shown to be a tetramer composed of two pairs of nonidentical subunits ($\alpha_2\beta_2$) (Berg et al., 1979). Two forms of the α subunit have been observed in immunoprecipitates of embryonic chick tendon cell homogenates (Berg et al., 1980); both forms were found to be present in tetrameric enzyme. Only one of these forms, α, is found in enzyme purified from 13-day-old whole chick embryos (WCE).[1] The α subunit contains at least two residues of *N*-acetylglucosamine and a larger amount of mannose (Berg

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[1] Abbreviations: Endo H, endo-β-*N*-acetylglucosaminidase H; Con A, concanavalin A; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CEF, chick embryo fibroblast; WCE, whole chick embryo; DTT, dithiothreitol; PPO, 2,5-diphenyloxazole, Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; RNase, ribonuclease.

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et al., 1979), a composition typical of the "high-mannose" class of asparagine-linked oligosaccharides. The other α-like subunit, α', was electrophoretically separated from the α in PAGE in SDS (Berg et al., 1980). Its carbohydrate composition has not previously been determined.

The glycosylation of Asn residues of proteins occurs in the endoplasmic reticulum (Schachter, 1974; Vijay et al., 1980; Hanover & Lennarz, 1980; Godelaine et al., 1982) by the transfer of a glucosylated oligosaccharide from dolichyl pyrophosphate [Chapman, 1978; Henner et al., 1981; for review, see Parodi & Leloir (1979)]. Soon after the oligosaccharide is linked to the protein, glucose residues are removed by membrane-bound enzymes, which appear to reside in the rough endoplasmic reticulum [for reviews, see Snider & Robbins (1981), Murphy & Spiro (1981), Hubbard & Ivatt (1981), Kornfeld (1982), Gogstad & Helgeland (1982), and Bergman & Kuehl (1982)]. Further processing of complex type oligosaccharides entails the removal of up to six mannose residues, some of which may be removed in the endoplasmic reticulum (Hercz et al., 1978; Godelaine et al., 1981) while others are trimmed in the golgi (Tabas & Kornfeld, 1979). Our purpose in characterizing the oligosaccharide chains of prolyl hydroxylase was 2-fold: first, to determine the class or type of carbohydrate unit found on an enzyme residing in the endoplasmic reticulum and, second, to clarify the relationship between α and α'.

Experimental Procedures

Materials

Thirteen-day-old and 17-day-old chick embryos were obtained from a local hatchery. Con A–Sepharose was purchased from Pharmacia. Endo H was purified from *Streptomyces plicatus* as previously described (Tkacz, 1978). A modification of the method of Snaith & Levvy (1968) was used to prepare jack bean α-mannosidase (Tkacz & Herscovics, 1975). Exoglycosidases in the α-mannosidase preparation were assayed at 30 °C by the procedures of Li & Li (1972). An amount of the preparation containing 1 unit of α-mannosidase had less than 10^{-5} unit of β-glucosidase, α-glucosidase, or α-galactosidase activity, less than 10^{-4} unit of β-mannosidase, β-galactosidase, or β-*N*-acetylglucosaminidase activity, and less than 10^{-3} unit of acid phosphatase activity. A crude mixture of α-mannosidase, α-galactosidase, and α-*N*-acetylglucosaminidase was obtained by extraction of jack bean meal. Bovine pancreatic ribonuclease B was purchased from Worthington and then further purified by affinity chromatography on Con A–Sepharose (Tkacz, 1978). Bio-Gel P2 (100–200 mesh), Bio-Gel P6 (200–400 mesh), and Bio-Gel P4 (200–400 mesh) were obtained from Bio-Rad Laboratories. ^{14}C-Labeled amino acid mix and carrier-free sodium [^{3}H]-borohydride were obtained from New England Nuclear. Methyl α-D-mannopyranoside was obtained from Sigma and *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories. Maltose, raffinose, and stachyose used for P6 calibration standards were a gift from Dr. Gad Avigad, University of Medicine and Dentistry of New Jersey, Piscataway, NJ. *N*-Acetylglucosaminitol was a gift from Dr. Christopher Warren, Massachusetts General Hospital, Boston, MA. All other reagents were obtained in highest purity from commercial sources.

Methods

Isolation of Prolyl Hydroxylase. Prolyl hydroxylase from 13-day-old chick embryos and from tendon cells (i.e., fibroblasts) of 17-day-old chick embryos was purified by using an affinity chromatographic procedure (Berg & Prockop, 1973; Tuderman et al., 1975). A further modification of the method involved the use of DEAE-cellulose to separate polyproline from the enzyme in the final step of the procedure (Kedersha & Berg, 1981). Purity of the enzyme was determined by PAGE in SDS; its concentration was measured by absorbance using 1% A_{230} of 77.3 (Berg et al., 1977).

Chick–tendon prolyl hydroxylase was purified from freshly isolated fibroblasts obtained from the leg tendons of 17-day-old chick embryos by enzyme digestion (Kao et al., 1977). Cells were subsequently incubated in modified krebs medium II (Dehm & Prockop, 1971) with labeled ^{14}C amino acid mix for 4 or 6 h; the cells were separated from the medium by centrifugation and frozen. Cells were later thawed, homogenized in buffer containing 0.01 M Tris-HCl and 0.1 M glycine, pH 7.9 at 4 °C, containing 0.2 M NaCl, 1.5 μM DTT, and 0.5% Nonidet P40 detergent, and centrifuged at 20000g for 30 min. The supernatant was applied to an affinity column and purified as described previously. The volume was adjusted before homogenization to give a concentration of 100×10^6 cells per mL.

Peptide Maps of Chick and Tendon Prolyl Hydroxylase. Freshly isolated fibroblasts were prepared and incubated in modified Krebs II media (Kao et al., 1977) without glucose but supplemented instead with 5 mM sodium pyruvate and 10 μg of sodium ascorbate/mL. These cells were incubated at a concentration of 10^7 per mL with either 4 μCi of [^{3}H]-mannose/mL or 0.2 μCi of [^{14}C]mannose/mL, at 37 °C for 4 or 6 h, and then treated as described above. Affinity-purified, labeled enzyme was treated with SDS and subjected to PAGE in SDS as described previously (Kao et al., 1977). After brief staining with Coomassie brilliant blue G, the α bands (including both α and α' species in the tendon cell enzyme) were cut out and subjected to peptide mapping according to the procedure of Cleveland et al. (1977) using 200 ng of *S. aureus* protease per well and digesting for 45 min at room temperature.

Con A Chromatography and Polyacrylamide Slab Gel Electrophoresis. Five hundred micrograms of purified prolyl hydroxylase was digested with 3.75 μg of Endo H in 50 mM sodium citrate, pH 7.0, containing 100 mM NaCl. The reaction was stopped by adding an equal volume of 0.01 M Tris-HCl buffer, pH 7.8 containing 0.1 M glycine and 0.2 M NaCl to raise the pH and thereby inactivate the Endo H. The mixture was then applied to a Con A–Sepharose column with a bed volume of 0.5 mL. The column was washed with approximately 8 mL of buffer and then eluted with buffer containing 10% methyl α-D-mannopyranoside. The optical density at 230 nm of each fraction was measured, and enzyme activity was assayed as described (Kao et al., 1975). To demonstrate that the reaction with Endo H had gone to completion, samples of prolyl hydroxylase were taken at various times during the digestion and subjected to PAGE in SDS by using a 10% polyacrylamide-separating gel as described previously (Kao et al., 1977).

Sodium [^{3}H]Borohydride Reduction. Five milligrams of chick prolyl hydroxylase was digested with 7.5 μg of Endo H in 2 mL of 50 mM sodium citrate buffer containing 100 mM NaCl, pH 6.5, at 30 °C for 12 h. To prepare a reference oligosaccharide, 3.5 mg of ribonuclease B were digested under identical conditions. Samples containing only prolyl hydroxylase, ribonuclease B, or Endo H were incubated under similar conditions as controls. The reactions were stopped by the addition of sufficient borate buffer (50 mM sodium borate, and 100 mM sodium chloride, pH 10.5) to raise the pH of the reaction mixture to 9.5. Each reaction mixture was reduced

with 250 μCi of sodium [^{3}H]borohydride for 4 or 13 h at 30 °C. The unreacted borohydride was subsequently removed by chromatography on a 48 × 0.9 cm column of Bio-Gel P 20 (100–200 mesh) equilibrated with 50 mM sodium borate and 100 mM sodium chloride, pH 8.0. The void volume fractions for each sample were pooled, and to destroy borohydride, the pH of the sample was adjusted to 5.0 by the addition of 0.5 mL of 0.1 M ammonium acetate followed by titration with acetic acid. The samples were incubated at room temperature for 1 h to precipitate the cleaved protein and then centrifuged for 5 min at 8000*g*. The same procedure was followed by using enzyme prepared from freshly isolated fibroblasts, with the following exceptions: only 1.2 mg of tendon cell prolyl hydroxylase was digested with 9.9 μg of Endo H for 3.5 h at 30 °C. Borohydride reduction was continued for 2 h at 30 °C.

Oligosaccharides labeled in vivo with radiolabeled sugars were reduced by using unlabeled sodium borohydride under the same conditions as described above. However, the subsequent separation of free borohydride in Bio-Gel P2 was omitted.

In initial experiments chromatography on P4 resulted in a number of peaks due to the reduction of amino acids in the polypeptide by [^{3}H]borohydride. This was not a problem with the material from 13-day-old chick embryos because the much higher protein concentration caused virtually all of the protein to precipitate when the sample was acidified and centrifuged prior to gel filtration. This was accompanied by a 90% precipitation of label, and the acidified pellet revealed labeled prolyl hydroxylase by PAGE in SDS (data not shown). In the tendon enzyme sample, no radioactivity or protein precipitated. When examined by chromatography on a P4 column, several broad peaks of labeled material were obtained. This problem was circumvented by applying the entire sample to a Con A column (same size and conditions as described above). The bound material was eluted, concentrated with a gentle stream of nitrogen gas, and then applied to the Bio-Gel P4 column.

Chromatography of the Oligosaccharides and Digestion with α-Mannosidase. The supernatants from each sample above were applied to either a 1 × 114 cm column of Bio-Gel P6 (200–400 mesh) or a similar column of Bio-Gel P4 (200–400 mesh), equilibrated and eluted with 0.1 M sodium acetate buffer, pH 5.0 (Etchison et al., 1977; Tabas et al., 1978). The purified oligosaccharide peaks thus obtained were pooled, lyophilized, and dissolved in 0.1 N acetic acid. Methanol was repeatedly added and removed under a stream of N_2 in order to remove any traces of borate. Samples were then evaporated to dryness and suspended in 50 mM sodium citrate buffer, pH 4.5. Each sample was digested with 7.3 units of purified α-mannosidase for 48 h at 37 °C overlaid with 50 μL of toluene and then subjected to chromatography on the same Bio-Gel column. The elution positions of raffinose and stachyose standards on the P6 column were determined by the phenol–sulfuric acid method (Dubois et al., 1956). Fractions containing radioactivity were pooled and lyophilized. The material was then examined by paper chromatography (Chen et al., 1975) using a 1-butanol/pyridine/water (6/4/3, v/v) solvent system. Standards of maltose and *N*-acetylglucosaminitol were detected with periodate–benzidine (Cifonelli & Smith, 1954). The radioactive product was located by cutting the chromatogram into 1-cm strips, eluting each strip with 1 mL of H_2O, and counting the eluate by liquid scintillation in Aquasol (New England Nuclear). The limit α-mannosidase digest of the reduced RNase oligosaccharide eluted slightly behind the maltose standard as does β-mannosyl-*N*-acetylglucosaminitol in the solvent system employed (Chen et al., 1975).

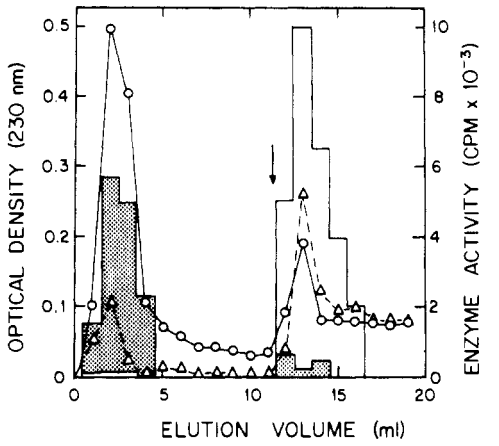

FIGURE 1: Concanavalin A–Sepharose chromatography of native and Endo H cleaved WCE prolyl hydroxylase. Five hundred micrograms each of native and Endo H digested prolyl hydroxylase was applied separately to a concanavalin A–Sepharose column with a bed volume of 0.5 mL and equilibrated with 0.01 M Tris-HCl, pH 7.8, containing 0.2 M NaCl and 0.1 M glycine. The column was briefly washed and then eluted with 10% methyl α-D-mannopyranoside. The recovery of enzyme activity from the column was greater than 90%. (△) indicates OD_{230} of native enzyme; (○) indicates OD_{230} of the Endo H digested enzyme. Open bars show enzyme activity of native prolyl hydroxylase, shaded bars indicate the activity of the Endo H treated enzyme.

Other Procedures. Slabs were prepared for fluorography by two 30-min incubations in 500 mL of dimethyl sulfoxide (Me_2SO) prior to a 3-h incubation in a PPO–Me_2SO solution (40 g of PPO in 150 mL of Me_2SO). The fluor was precipitated in the gel by a 1-h wash in running deionized water. Gels were dried and exposed to Kodak X-O-matic film at −70 °C for 1–3 weeks.

RESULTS

Effect of Endo H Digestion on Chick Embryo Prolyl Hydroxylase. Whole 13-day-old chick embryos (hereafter termed WCE) contain prolyl hydroxylase with α subunits that are mannosylated and that bind to Con A (Berg et al., 1979). As shown in Figure 1, reactivity with Con A–Sepharose is abolished when WCE prolyl hydroxylase is treated with Endo H, indicating that all accessible α-linked mannose can be completely removed from the glycoprotein by this glycosidase. In light of the specificity of Endo H (Tai et al., 1977), the result implies that carbohydrate–protein linkages of the (*N*,*N*′-diacetylchitobiosyl)asparaginyl type are present in native prolyl hydroxylase. The removal of sugar did not significantly alter the catalytic activity of prolyl hydroxylase (Figure 1).

When the treated prolyl hydroxylase was analyzed by polyacrylamide slab gel electrophoresis in SDS, it was found that Endo H caused an increase in mobility of the α subunit to a form designated as α_0 but did not affect the mobility of the β subunits (Figure 2, cf. lanes 1 and 7). This is in agreement with previous results that indicated that mannose and *N*-acetylglucosamine are present in the α subunits but not in the β subunit (Berg et al., 1979). From the Con A binding results (Figure 1) it is clear that α_0 (Figure 2) represents an α subunit containing no mannose that is accessible to Con A. Incomplete digestion of WCE prolyl hydroxylase with Endo H yielded preparations with only two protein species migrating in the α-subunit region, viz., the native α subunit and the α_0 form (Figure 2, lanes 2–6). Species with mobilities between those of the native subunit α and those of α_0 should have been

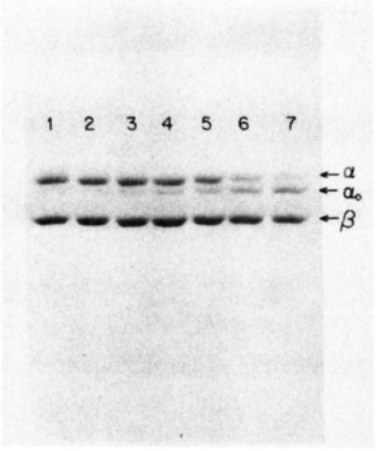

FIGURE 2: Polyacrylamide gel electrophoresis in SDS of the WCE enzyme, showing the altered mobility of the α subunit as the result of Endo H digestion. A reaction mixture containing 5 mg of prolyl hydroxylase was incubated for 12 h at 30 °C with 7.5 μg of purified Endo H. Aliquots were taken for electrophoresis at the following times: Lane 1, 0 min; lane 2, 5 min; lane 3, 15 min; lane 4, 30 min; lane 5, 60 min; lane 6, 90 min; lane 7, 120 min.

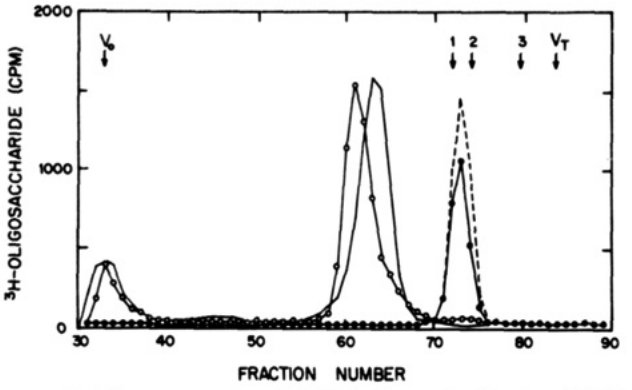

FIGURE 3: Chromatography of labeled oligosaccharides from WCE prolyl hydroxylase and ribonuclease B. The oligosaccharides were obtained as described under Experimental Procedures and applied to a 1 × 115 cm column of Bio-Gel P6 equilibrated with 0.1 M ammonium acetate, pH 5.0. One-milliliter fractions were collected, and ^3H_2O was added to each sample to mark the total volume of the column. (O) Prolyl hydroxylase oligosaccharide; (—) ribonuclease B oligosaccharide; (●) prolyl hydroxylase oligosaccharide after α-mannosidase digestion; (---) ribonuclease β oligosaccharide after α-mannosidase digestion. V_0, the elution position of blue dextran; V_t, the elution position of ^3H_2O. The elution positions of the saccharide standards are designated: 1, stachyose; 2, raffinose; 3, maltose.

observed if each native WCE subunit contained more than one oligosaccharide (see below).

Gel Filtration of Oligosaccharide from WCE Prolyl Hydroxylase. For further characterization of the oligosaccharide from the α subunit, samples containing the carbohydrate and protein products of Endo H digestion were reduced with sodium [^{3}H]borohydride. Chromatography using Bio-Gel P2 was employed to separate the reduced products from the residual borohydride. When acidified and cleared of protein precipitate by centrifugation and subjected to chromatography on Bio-Gel P6, the reduced products were separated into two fractions, one in the void volume and another that appeared approximately midway between the void volume and the volume at which ^3H_2O eluted (Figure 3). The second peak was identified as the oligosaccharide released by the glycosidase: it was not obtained when the glycoprotein was incubated in the absence of Endo H prior to reduction or when Endo H itself was reduced. Similar material was generated by Endo H treatment of ribonuclease B; this material likewise

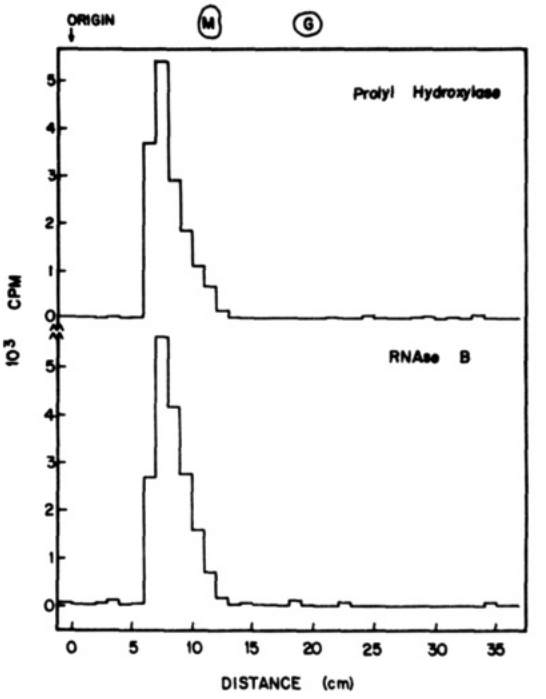

FIGURE 4: Paper chromatography of the limit digests obtained by using α-mannosidase on the labeled oligosaccharides as described under Experimental Procedures and also as shown in Figure 3. M, the elution position of maltose; G, the elution position of *N*-acetylglucosaminitol.

was not present in a reduced control of ribonuclease B incubated without Endo H (Figure 3).

The material from the void volume was the protein product of Endo H action that did not precipitate upon acidification of the sample. There are several lines of experimental evidence indicating that this material did not contain cleaved carbohydrates: (a) the material appeared in the void volume whether or not the glycoprotein had been incubated with Endo H; (b) the material was apparently unaltered by treatment with purified α-mannosidase or a mixture of glycosidases (including α-mannosidase, β-galactosidase, and β-*N*-acetylglucosaminidase) but was sensitive to trypsin; (c) the radioactivity could be removed by reacting the material with 2-mercaptoethanol. The void volume material did not appear to be carbohydrate in nature and was not analyzed further.

Endo H digests of ribonuclease B, a glycoprotein molecule with a single carbohydrate moiety (Tarentino et al., 1970), generated material eluting in the void volume as well as material eluting near the oligosaccharide obtained from prolyl hydroxylase (Figure 3). From the elution patterns shown in Figure 3, it is clear that the oligosaccharide cleaved by Endo H from ribonuclease B is slightly smaller than that released from prolyl hydroxylase. However, when exhaustively digested with α-mannosidase, both oligosaccharides were converted to the same smaller fragment, a species that elutes between unreduced tri- and tetrasaccharide standards (raffinose and stachyose) (Figure 3).

Since unreduced sugars are not appropriate for calibration of gel permeation columns when the size of reduced saccharides is to be estimated (Liang et al., 1979), the α-mannosidase digests were subjected to paper chromatography in order to characterize the products (Figure 4). Both α-mannosidase digestion products migrated slightly more slowly than maltose, and the same is true of β-mannosyl-*N*-acetylglucosaminitol in the solvent system employed (Chen et al., 1975). The complete conversion of both oligosaccharides to an apparent disaccharide by the action of α-mannosidase im-

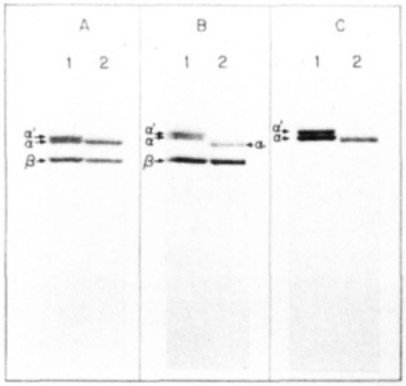

FIGURE 5: (A) Polyacrylamide gel electrophoresis in SDS stained with Coomassie brilliant blue R, revealing the differences between affinity-purified WCE enzyme and affinity-purified CEF enzyme. Lane 1, CEF enzyme containing two forms of α subunits, designated α and α'; lane 2, WCE enzyme containing a single form of α subunit. (B) Polyacrylamide gel electrophoresis in SDS showing the effect of Endo H on the mobility of CEF α and α'. Lane 1, native CEF enzyme; lane 2, enzyme after treatment with Endo H. (C) Fluorogram of polyacrylamide gel electrophoresis in SDS showing the effect of α-mannosidase on the mobility of the two forms of CEF α subunits. Lane 1, native CEF enzyme; lane 2, enzyme after treatment with α-mannosidase. Enzyme was labeled with ^{14}C amino acid mix, purified as described under Methods, and digested with α-mannosidase for 48 h at 37 °C under toluene. It was necessary to visualize the progress of the reaction by fluorography as the α subunits comigrate with α-mannosidase under the conditions used here. The β subunit is not labeled with amino acid mix due to the existence of a large pool of preformed material (Berg et al., 1980).

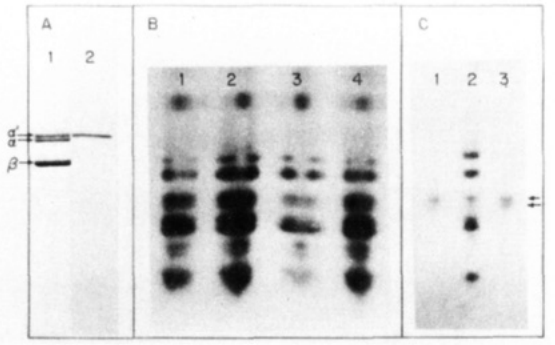

FIGURE 6: (A) Polyacrylamide gel electrophoresis of CEF enzyme radiolabeled with [^{3}H]mannose. Lane 1, stained protein; lane 2, fluorography of in vivo radiolabeled enzyme (see text). (B) Comparison of peptides from α of WCE enzyme with peptides of α and α' from CEF enzyme. After brief staining with Coomassie brilliant blue G, bands were cut from a 10% SDS–PAGE slab gel, briefly equilibrated with sample buffer, and applied to a 15% polyacrylamide gel as described previously (Cleveland et al., 1977). Buffer containing *S. aureus* V8 protease was layered over each sample and an electric field (40 mA) applied until the tracking dye reached the interface between stacking and separating gel. The current was shut off and digestion allowed to proceed for 45 min, after which the electrophoresis was completed and the gel was stained with Coomassie brilliant blue R. Lane 1, CEF α digest; lane 2, CEF α' digest; lane 3, WCE α digest; lane 4, CEF α digest. (C) Fluorography of polyacrylamide gel electrophoresis. The sample in each lane was digested with 200 ng of *S. aureus* V8 protease. Lane 1 contains the digest of [^{3}H]-mannose-labeled prolyl hydroxylase α subunits (both α and α'); lane 2, the same, only labeled with amino acid mix; lane 3, the same, only labeled with [^{14}C]mannose.

plies the absence of covalently linked glucose, *N*-acetylglucosamine, or other complex type sugars at their nonreducing termini.

Comparison of WCE and CEF Enzymes and Their Oligosaccharides. Previous studies revealed that immunoprecipitates of tendon cell homogenates using antibodies specific for the β subunit of prolyl hydroxylase (therefore precipitating α subunits present in tetramers) contained *two* α-like polypeptides (Berg et al., 1980). These two α polypeptides are also found in enzyme purified from freshly isolated tendon fibroblasts (CEF) by affinity chromatography. One of them behaves as the α subunit of the WCE enzyme on PAGE in SDS, whereas the other (α') is slightly less mobile (Figure 5A). Both the α and the α' of CEF enzyme showed increased mobility on PAGE in SDS after Endo H treatment (Figure 5B), yielding a single band. This result suggests that α and α' differ solely in their carbohydrate composition. Similar results were obtained with α-mannosidase (Figure 5C).

Linear *S. aureus* V8 protease maps (Cleveland et al., 1977) were made in order to determine the degree of structural similarity between α and α' and to identify the glycopeptides of each species. In order to completely separate α from α', very long 10% gels were made to allow the two α bands to be excised separately. The resulting peptide maps of the separated α species revealed apparent homology between α and α' (Figure 6B, lanes 1 and 2). The WCE peptides (Figure 6B, lane 3) yielded a similar but not identical pattern as compared with the peptides of the CEF α and α'.

To identify which, if any, of the peptides were glycopeptides, CEF's were labeled with either [2-^{3}H]mannose, [^{14}C]mannose, or ^{14}C amino acid mix prior to the isolation of prolyl hydroxylase by affinity chromatography. Surprisingly, only the larger α' was found to incorporate measurable mannose radioisotope during a 4.5-h incubation (Figure 6A), although both α species contain mannose as indicated by the altered electrophoretic mobility of both species after treatment with Endo H or α-mannosidase (Figure 5B,C). The inability of certain glycoproteins to incorporate exogenously supplied radiolabeled mannose has been reported elsewhere (Gottlieb & Wallace, 1982); we present further biosynthetic data on this in the following paper (Kedersha et al., 1985). We exploited this property in the following manner: enzyme labeled in vivo was purified, the distribution of the radiolabeled mannose was shown to be restricted to the α' subunit by SDS–PAGE (Figure 6A), and the bands were excised and subjected to S. *aureus* peptide mapping in the second dimension. Fluorography revealed the presence of two mannose-radiolabeled peptides (see Figure 6C, lanes 1 and 3). It is obvious that each radiolabeled species must be derived solely from α'. This result indicated that two glycopeptides were derived from α' and suggested that native α' contained two oligosaccharides. To confirm this, CEF enzyme containing unlabeled α and [2-^{3}H]mannose-labeled α' were digested with Endo H for various times and subjected to PAGE in SDS in order to ascertain whether the removal of labeled carbohydrate by this glycosidase occurs as a one-step (single oligosaccharide) or two-step (two oligosaccharides) process. Protein staining of the electrophoretogram (Figure 7A) shows that both α and α' are converted to products of the same apparent molecular weight by Endo H action. The fluorogram of the same gel (Figure 7B) reveals that the removal of labeled carbohydrate is accompanied by the transient appearance of a labeled species smaller than α' but larger than its fully deglycosylated product visible only by the protein stain (Figure 7A). The two-step alteration of mobility is consistent with the conclusion that two oligosaccharides are present on each native α' subunit.

When unlabeled CEF enzyme was completely cleaved by Endo H and the carbohydrate subsequently reduced by sodium [^{3}H]borohydride, the resulting labeled carbohydrate (after purification and concentration on a small Con A column) could be nearly completely separated into two oligosaccharides by chromatography on Bio-Gel P4 (see Figure 8). Comparison

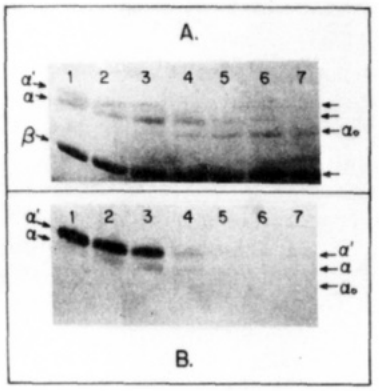

FIGURE 7: (A) Polyacrylamide gel electrophoresis of purified CEF prolyl hydroxylase labeled with [2-^{3}H]mannose and digested with Endo H. Thirty micrograms of enzyme was digested with 0.025 unit of Endo H at 37 °C, and aliquots were taken at various intervals. The gel was stained for protein with Coomassie brilliant R. Lane 1, 0 min; lane 2, 10 min; lane 3, 30 min; lane 4, 60 min; lane 5, 120 min. (B) Fluorogram of the same gel as in (A).

of the label contained in the partially separated peaks indicated that the ratio of larger to smaller oligosaccharide was 2:1. The smaller oligosaccharide coeluted with that released from WCE prolyl hydroxylase, while the larger oligosaccharide coeluted with the sole species obtained when in vivo [^{3}H]mannose labeling of prolyl hydroxylase was used prior to the isolation of the oligosaccharide (see below). It therefore appears likely that only Man_8 oligosaccharides are derived from α'. As the starting CEF material contained α and α' in equal quantities, this 2:1 ratio of borohydride-reduced oligosaccharides provides further support for the contention that α' contains two oligosaccharides as compared to α which contains one. Each oligosaccharide was susceptible to the action of α-mannosidase, yielding a single peak on P4 that corresponded to β-mannosyl-*N*-acetylglucosaminitol.

To determine the precise number of mannose residues in the oligosaccharides of α', cells were incubated for 5 h in the presence of [2-^{3}H]mannose, labeling only α' (see Figure 7A). The enzyme was isolated by affinity chromatography, treated with Endo H as described above, and then reduced with unlabeled sodium borohydride. Chromatography on a P4 column was employed to isolate labeled oligosaccharides (Figure 9). Following the digestion of the recovered oligosaccharide with α-mannosidase, the reaction products were separated by chromatography on Bio-Gel P4, and the radioactivity in the free mannose was compared with that eluting as mannosyl-*N*-acetylglucosaminitol. Ratios of 7.21:1 and 6.94:1 were obtained in two separate experiments, demonstrating that the intact oligosaccharide derived from α' contained eight mannosyl residues.

The oligosaccharide obtained from α' that had been labeled in vivo (Figure 9) appeared to be a quite homogeneous species that eluted from P4 in the same position as the larger of the two [^{3}H]borohydride-labeled oligosaccharides obtained from the CEF enzyme (Figure 8), with a trace of label eluting at the WCE oligosaccharide position. It seems likely that α' contains almost exclusively oligosaccharides with eight mannosyl residues. The oligosaccharide obtained from WCE enzyme on P6 (Figure 3) eluted from P4 in the position of the smaller of the two [^{3}H]borohydride-labeled oligosaccharides from the CEF enzyme (Figure 8, peak II), showing that the α subunits from each source are glycosylated with moieties of the same size. The number of mannose residues in the oligosaccharide derived from WCE would seem to be less than eight but larger than six, the number of mannose residues in the oligosaccharide of ribonuclease B (Tarentino et al., 1970). A value of seven is consistent with the molecular weight derived from a plot of log molecular weight vs. elution position on P4 (Figure 8, insert).

DISCUSSION

Previous observations (Berg et al., 1980) have indicated that two distinct forms of prolyl hydroxylase exist. The enzyme found in immunoprecipitates of tendon cell lysates possesses

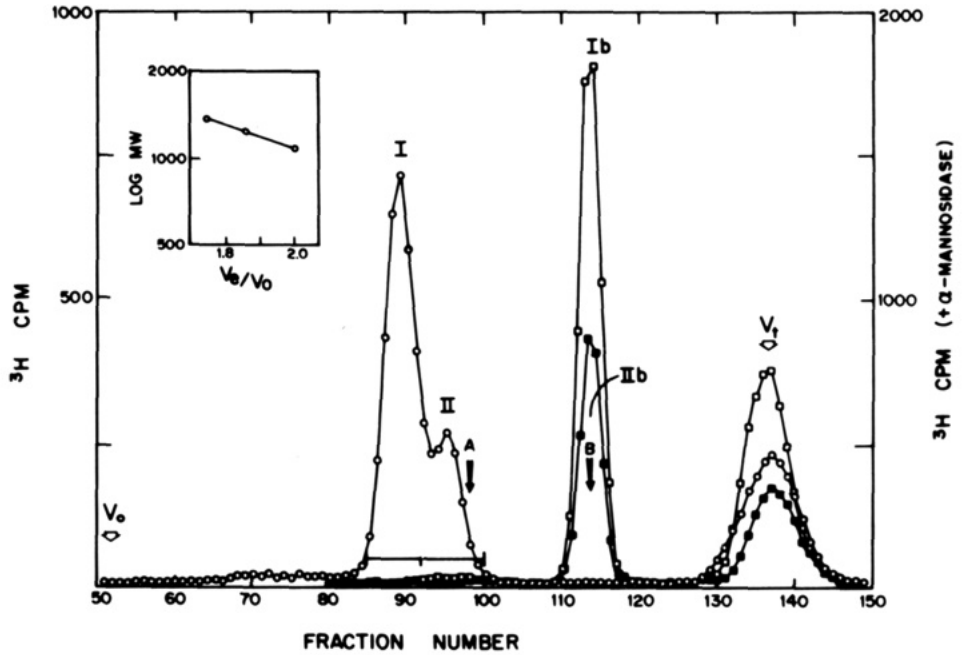

FIGURE 8: Chromatography on Bio-Gel P4 of the [^{3}H]borohydride-labeled oligosaccharide from CEF enzyme before and after α-mannosidase digestion. For conditions see Experimental Procedures. Circles represent labeled material before α-mannosidase digestion. Bars indicate fractions pooled and subjected to α-mannosidase. Unfilled squares indicate pool I peak after digestion with α-mannosidase; filled squares indicate material from pool II after digestion with α-mannosidase. A indicates the elution position of ribonuclease B oligosaccharide; B indicates the corresponding material digested with α-mannosidase. Open arrows mark the void volume, V_0, and total volume, V_t, respectively. (insert) Calculated molecular weight of I, II, and ribonuclease B oligosaccharides plotted vs. V_e/V_0. The oligosaccharide of ribonuclease B was assumed to be $Man_6GlcNAc$-OH (Tarentino et al., 1970). A linear relationship was found only when Man = 8 for I and Man = 7 for II.

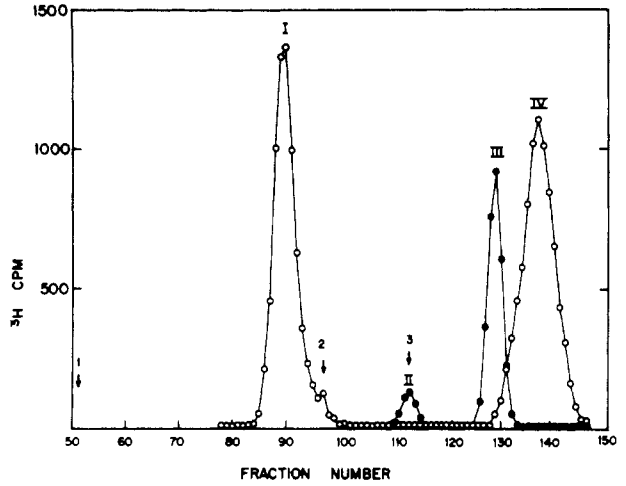

FIGURE 9: Chromatography on Bio-Gel P4 of [2-^{3}H]mannose-labeled oligosaccharide of CEF prolyl hydroxylase released by treatment with Endo H. Open circles indicate Endo H treated enzyme (^3H_2O was added as a marker); closed circles indicate material from peak I after α-mannosidase digestion. Arrow 2 indicates elution position of [^{3}H]borohydride-labeled oligosaccharide from α subunit oligosaccharide (see text); arrow 3 indicates elution position of α-mannosyl-*N*-acetylglucosaminitol. Peak III comigrates with free mannose. The ratio of peak I to peak III was found to be 7 to 1.

two different forms of α subunit which can be resolved by PAGE in SDS. Here we show that this form of prolyl hydroxylase can also be purified from tendon cells by the same affinity column procedure used to purify the enzyme from whole chick embryos and that like the immunoprecipitated enzyme it contains two types of α subunit. Endo H digestion followed by PAGE in SDS gave polypeptide chains of the same size derived from either α or α', suggesting that differences in the size and/or number of oligosaccharide chains in the two α subunits accounted for their electrophoretic separation.

Since the WCE enzyme was both structurally less complex (i.e., was composed of α and β but no α' subunits) and was available in larger quantities, it was the focus of our initial carbohydrate analysis. Its susceptibility to Endo H, subsequent failure to bind Con A, and altered mobility on PAGE in SDS after Endo H treatment all support the conclusion that the α subunit contains a single "high mannose" oligosaccharide joined to the polypeptide chain through a di-*N*-acetylchitobiosyl linkage. Our data also show that there are evidently no substituents present on the oligosaccharide to protect the α-linked mannosyl residues from exo attack by jack bean α-mannosidase. The number of mannosyl units contained in its oligosaccharide appears to be at least one more than the carbohydrate moiety of ribonuclease B which contains six mannosyl residues (Tarentino et al., 1970). Barring major differences in the hydrodynamic shapes of the two oligosaccharides, we conclude that the oligosaccharide of the α subunit of WCE prolyl hydroxylase contains at least seven mannosyl residues. This value for the amount of mannose on prolyl hydroxylase is less than previously obtained (Berg et al., 1979) when gas chromatography on intact α and β subunits was used. The previously reported higher values may have been caused by trace contaminants as in the present studies we have obtained more highly purified enzyme using an improved purification procedure (Kedersha & Berg, 1981) that employs an additional chromatographic step. Additionally, the current studies consider only Endo H cleaved material rather than hydrolysates of entire α and β subunits as was previously done (Berg et al., 1979). It is also possible that some non-Endo H sensitive mannose residues are present in the α subunit which contributed to the higher values reported previously, although the Endo H treated enzyme no longer interacts with Con A (Figure 1).

The oligosaccharide derived from the α subunit from CEF enzyme appears to be similar to that purified from WCE containing seven mannose residues, while the larger form α' was observed to contain two oligosaccharides each containing eight mannose residues. The separation of CEF α and α' on SDS–PAGE is apparently a function of their different carbohydrate content, and this mobility difference is eliminated upon treatment of the CEF enzyme with either Endo H or α-mannosidase. There are several indepedent lines of evidence indicating that α' possesses two oligosaccharides: (1) Labeled [^{3}H]borohydride preparations of total CEF oligosaccharides indicate that there are twice as many Man_8 oligosaccharides as there are Man_7 oligosaccharides. A 2:1 ratio of Man_8 to Man_7 oligosaccharides generated from a 1:1 ratio of α:α' requires that each α' contain two oligosaccharides while α contains one. This is consistent with the observed mobility differences between α', α, and α_0 on SDS–PAGE (see Figure 5B). (2) As only α' is labeled by [^{3}H]mannose during any of the labeling periods used (see Figure 6A), the two [^{3}H]-mannose-labeled peptides generated by V8 protease (Figure 6C) both derive from α' which indicates that the native α' contains two glycopeptides. (3) The treatment of [^{3}H]-mannose-labeled α' with Endo H is a two-step process, generating a labeled intermediate species of greater mobility than α' prior to the removal of all labeled mannose to yield an unlabeled α_0.

While the unexplained mannose labeling of α' but not α suggests that α' may be an untrimmed form of α, other evidence indicates that this is not the case, as pulse-labeling of the enzyme with radiolabeled amino acids demonstrates that the two subunits are synthesized simultaneously and that no precursor–product relationship exists (not shown). The kinetics of oligosaccharide processing in vivo are quite rapid, and they are mediated to some extent by the translocation of glycoproteins from one subcellular compartment to another. Prolyl hydroxylase is believed to reside solely in the rough endoplasmic reticulum; therefore, enzyme labeled in vivo for 4.5 h should be representative of the steady state level of α' with regard to oligosaccharide processing. The use of in vivo mannose-radiolabeled α' in these structural studies is therefore appropriate; further studies on the biosynthesis of the two forms of CEF are considered separately [see Kedersha et al. (1985)].

Both CEF α and α' oligosaccharides were completely digested by the action of α-mannosidase to a dissacharide-like species, which implies that α' does not contain any terminal glucosyl residues. The accepted dolichol-mediated N-linked oligosaccharide biosynthesis involves a $(GlcNAc)_3(Man)_9$-$(Glc)_3$ species as the transfer oligosaccharide, and this species has indeed been shown to operate in chick embryo fibroblasts (Hubbard & Robbins, 1980). The heterogeneity of oligosaccharides observed between α and α' can either be explained by differences in processing of a common precursor or by differences in the biosynthesis itself. Should the former be the case, the Man_7 and Man_8 oligosaccharides found here on this rough endoplasmic reticulum enzyme suggest that processing enzymes capable of removing some mannosyl residues as well as glucosyl residues reside in this organelle, as suggested by the work of Hercz et al. (1978) and reviewed by Schachter (1981). However, our mannose-labeling results suggest that the latter possibility may be more likely, and we have investigated this further [see Kedersha et al. (1985)].

The finding that α contains one while α' contains two oligosaccharides can be viewed in several ways. One assumption is that the two forms possess identical primary sequence and that α represents an underglycosylated form of α' in which only one of two potential sites is glycosylated. Variable glycosylation of an identical polypeptide sequence occurs in bovine pancreatic RNase (reviewed by Kornfeld and Kornfeld, 1980), but with one species greatly predominating, unlike the 1:1 ratio of α to α' observed here. Alternatively, α and α' could represent two gene products, in which very slight differences in primary sequence, although insufficient to result in different *S. Aureus* linear peptide maps as shown here, result in two glycosylation sites on α' but only one on α. Preliminary results of cyanogen bromide peptide maps demonstrate that at least 3 peptides from α migrate faster in polyacrylamide gel electrophoresis in SDS than analogous peptides in α'. Furthermore, α and α' are resolved by isoelectric focusing and have p*I*s of 5.46 and 5.42, respectively (N. L. Kedersha and R. A. Berg, unpublished results). Since the sequence Asn-X-Thr/Ser is necessary for glycosylation, it is clear that a single amino acid change could alter the number of glycosylation sites on the polypeptide. From the available data, it appears likely that α and α' are homologous but contain slightly different amino acid sequences which are sufficient to account for their differences in glycosylation.

The availability of α or α' subunits is the limiting factor in the formation of active enzyme tetramers (Berg et al., 1980) and is therefore of possible regulatory significance in the biosynthesis of collagen (Prockop et al., 1976). The existence of two genetically distinct forms of α would enable separate transcriptional controls. Prolyl hydroxylase has been purified from a number of other sources such as L929 cells (Berg et al., 1980), newborn mice (N. L. Kedersha and R. A. Berg, unpublished results), and human liver (R. A. Berg and G. D. Benson, unpublished results); all yield enzyme that contains two forms of α subunits in a 1:1 ratio. The lack of α' in enzyme purified from whole chick embryos represents the exception rather than the rule; preliminary studies (N. Kedersha, unpublished results) suggest that tetramers containing α' are selectively lost or degraded during the fractionation procedures performed prior to affinity chromatography. As yet it has not been determined whether CEF tetramers consist of homomers ($\alpha_2\beta_2$ and $\alpha'_2\beta_2$) or of heteromers (α, α', β_2). The latter tetrameric association would provide a mechanism for the maintenance of the observed 1:1 ratio of α to α'.

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Registry No. Prolyl hydroxylase, 9028-06-2.

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Biosynthesis of Prolyl Hydroxylase: Evidence for Two Separate Dolichol-Mediated Pathways of Glycosylation†

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ABSTRACT: Prolyl hydroxylase is a glycoprotein containing two nonidentical subunits, α and β. The α subunit of prolyl hydroxylase isolated from 13-day-old chick embryos contains a single high mannose oligosaccharide having seven mannosyl residues. Two forms of α subunit have been shown to exist in enzyme purified from tendon cells of 17-day-old chick embryos, one of which (α) appears to be identical in molecular weight and carbohydrate content with the single α of enzyme from 13-day-old chick embryos, as well as another form (α') that contains two oligosaccharides, each containing eight mannosyl units [see Kedersha, N. L., Tkacz, J. S., & Berg, R. A. (1985) *Biochemistry* (preceding paper in this issue)]. Biosynthetic labeling studies were performed with chick tendon cells using [2-^{3}H]mannose, [6-^{3}H]glucosamine, [^{14}C(U)]mannose, and [^{14}C(U)]glucose. Analysis of the labeled products using polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that only the oligosaccharides on α' incorporated measurable mannose or glucosamine isotopes; however, both α subunits incorporated ^{14}C amino acid mix and [^{14}C(U)]glucose [metabolically converted to [^{14}C(U)]mannose] under similar conditions. Pulse–chase labeling studies using ^{14}C amino acid mix demonstrated that both glycosylated polypeptide chains α and α' were synthesized simultaneously and that no precursor product relationship between α and α' was apparent. In the presence of tunicamycin, neither α nor α' was detected; a single polypeptide of greater mobility appeared instead. Incubation of the cells with inhibitory concentrations of glucosamine partially depressed the glycosylation of α' but allowed the glycosylation of α. Therefore, although both α and α' are synthesized and glycosylated simultaneously, the former appears to be glycosylated exclusively with unlabeled mannose or labeled mannose derived from [^{14}C(U)]glucose but not [2-^{3}H]mannose whereas the latter readily utilizes [2-^{3}H]mannose, [6-^{3}H]glucosamine, or labeled mannose derived from [^{14}C(U)]glucose. These results suggest the existence of two separate pathways of oligosaccharide biosynthesis, both mediated by dolichol but differing in their ability to be labeled by exogenous sugars, in the size of the high mannose oligosaccharides ultimately present on prolyl hydroxylase, and in their susceptibility to inhibition by high levels of glucosamine.

The dolichol-mediated biosynthesis of N-linked oligosaccharides has in the past 10 years been the subject of intensive study [for reviews, see Parodi & Leloir (1979), Kornfeld & Kornfeld (1980), Struck & Lennarz (1980), Hubbard & Ivatt (1981), Snider & Robbins (1981), and Bergman & Kuehl (1982)]. It is generally accepted that these oligosaccharides are synthesized by the ordered stepwise transfer first of *N*-acetylglucosamine 1-phosphate and then *N*-acetylglucosamine, mannose, and glucose from their nucleotide or lipid donors to the lipid carrier–acceptor dolichol phosphate (Chapman et al., 1978; Vijay et al., 1980; Rearick et al., 1981; Hubbard & Robbins, 1980). In mammalian systems, the ultimate lipid-linked product is apparently $Glc_3Man_9GlnNAc_2$ (Chapman et al., 1979; Henner et al., 1981), which is transferred "en bloc" to recipient polypeptides. In chick embryo fibroblasts the pathway has been especially well characterized (Hubbard & Robbins, 1979; 1980).

The majority of oligosaccharide biosynthetic studies in vivo have relied heavily on the use of radioisotopes, usually [2-^{3}H]mannose. Such studies have led to the accepted view of

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