SUBCELLUAR SITES FOR SYNTHESIS OF CHONDROMUCOPROTEIN OF CARTILAGE

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ABSTRACT

Microsomes from embryonic cartilage have been subfractionated to yield smooth microsomes and rough microsomes. The in vitro enzymic activities involved in chondroitin sulfate biosynthesis have been assayed in these subfractions. The results demonstrate that all of the activities necessary for linkage to protein as well as for completion of the polysaccharide chain are present in both the rough and smooth fractions. Only in the case of the polymerization of *N*-acetylgalactosamine and glucuronic acid could enzyme assays be done independent of endogenous acceptor. This enzyme(s) was equally distributed between the rough and smooth fractions. The activities for the addition of xylose and galactose to protein were highest in the rough fraction while that for sulfation was highest in the smooth fraction. These findings suggest that polysaccharide chain-initiation occurs in the rough endoplasmic reticulum and that chain completion occurs in the smooth reticulum. This pattern is consistent with modern theories of synthesis, transfer, and export of extracellular macromolecules.

INTRODUCTION

Recent studies have in a large measure elucidated the pathway of biosynthesis of chondromucoprotein in embryonic chick cartilage. Cell-free particulate systems have been isolated which appear to promote the synthesis of the protein-polysaccharide linkage region (1, 2), the alternate addition of *N*-acetylgalactosamine and uronic acid (3, 4), and finally the transfer of sulfate to polysaccharide (5) from PAPS.[1] The enzymes involved in these syntheses have been localized to the microsomal fraction of homogenates.

The role of the rough and smooth endoplasmic reticulum, including the Golgi apparatus, in the synthesis, transfer, and export of extracellular proteins has been investigated (6, 7). Much less is known about the subcellular sites of synthesis of complex protein such as chondromucoprotein.

Radioautographic studies have been carried out in attempts to locate the sites of chondroitin sulfate synthesis (8–10). These studies have involved the localization of radioactivity following the introduction of labeled precursors in vivo. In general it has been concluded that addition of carbohydrate occurs in the Golgi apparatus.

The subcellular sites of the synthesis of liver glycoproteins have been studied by several techniques involving the introduction of labeled precursors in vivo followed by fractionation of subcellular components by methods previously

[1] Abbreviations used are as follows: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; APS, adenosine phosphosulfate; CS, chondroitin sulfate; CS-4-SO_4, chondroitin-4-sulfate; CS-4 hexa, chondroitin-4-sulfate hexasaccharide; CS-6-SO_4, chondroitin-6-sulfate; CS-6 penta, chondroitin-6-sulfate pentasaccharide; UDP-GlcUA, uridinediphosphate glucuronic acid; UDP-GalNAc, uridinediphosphate *N*-acetylgalactosamine; UDP-Gal, uridinediphosphate galactose; UDP-Xyl, uridinediphosphate xylose; CPC, cetylpyridinium chloride.

applied to liver cells (11–13). These methods resulted in the separation of the smooth and rough microsomes having morphological, chemical, and enzymatic differences. That the distribution of radioactivity in subcellular fractions was found to vary with time suggests the progression of product from rough to smooth endoplasmic reticulum.

The detailed structure of the linkage to protein of chondroitin sulfate (illustrated in Fig. 1) has recently been elucidated (14). It has also been demonstrated that biosynthesis proceeds by the stepwise addition of monosaccharide units to a protein acceptor. For these reasons, it seemed possible to examine for the first time the subcellular distribution of enzymatic activities involved in this biosynthetic sequence rather than merely to follow the fate of radioactivity after in vivo administration of labeled precursors. It is the purpose of this paper to report the results of such experiments. A preliminary report of somewhat similar studies has been presented by Suzuki et al. (15).

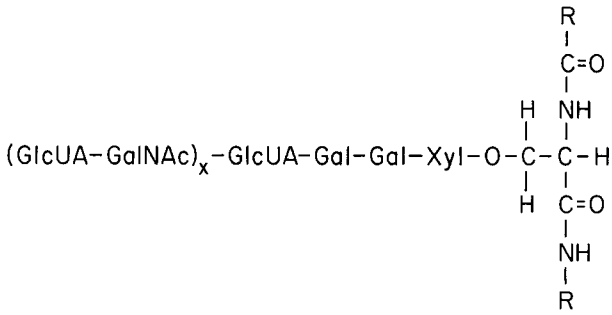

FIGURE 1 The structure of chondromucoprotein. The R groups represent the remaining protein core to which other carbohydrate side chains are attached by similar xylosyl serine bonds. The number of repeating disaccharides of the polysaccharide portion in mature cartilage is about 50.

MATERIALS AND METHODS

Reagents

The following uridinediphosphate sugars were obtained as described previously (2–4): UDP-GlcUA-^{14}C, UDP-GalNAc-^{3}H, UDP-Gal-^{14}C, UDP-GlcUA, UDP-GalNAc. UDP-Xyl-^{14}C was kindly prepared by Dr. D. S. Feingold. PAPS-^{35}S was prepared by using carrier-free $^{35}SO_4$ with a crude enzyme preparation derived from rat intestinal mucosa (16). Paper electrophoresis showed that the preparation contained 75% PAPS-^{35}S and 25% APS-^{35}S with no free sulfate.

Testicular hyaluronidase (20,000 IU/mg) was obtained from AB Leo, Hälsingborg, Sweden. Ribonuclease and papain were purchased from Worthington Biochemical Corp. (Freehold, N. J.). MES buffer (2-[*N*-morpholino]ethanesulfonic acid, pK_a 6.1) was purchased from Calbiochem (Los Angeles, Calif.). Yeast RNA was obtained from Nutrional Biochemicals Corporation (Cleveland, O.).

Carrier CS-4-SO_4 and CS-4 hexa were prepared as previously described (3, 4). CS-6 penta was prepared from a hyaluronidase digest of umbilical cord CS-6-SO_4 and showed chemical analyses and chromatographic properties consistent with a 6-O-sulfated pentasaccharide (4).

Buffers

Buffers were adjusted to pH 7.0 unless otherwise stated. Buffer A contained sucrose, 0.2 M; MES, 0.05 M; KCl, 0.05 M. Buffer B contained MES, 0.05 M; KCl, 0.05 M; $MnCl_2$, 0.003 M; cysteine, 0.006 M. Buffer C contained sucrose, 1.3 M; MES, 0.05 M; KCl, 0.05 M; CsCl, 0.015 M.

Preparation of Subcellular Fractions

Epiphyses of femurs and tibias from 13-day-old chick embryos were dissected free of soft tissues, chilled, and finely diced. The diced epiphyses were homogenized in four volumes of buffer A in a glass Potter-Elvehjem homogenizer with a motor-driven Teflon pestle by using 10–12 strokes with the pestle revolving at about 1000 rpm. The buffer salts were found to be necessary since homogenization in 0.25 M sucrose in the absence of salts resulted in a viscous suspension which could not be successfully centrifuged. The homogenate was centrifuged at 10,000 g[2] for 10 min. The supernatant solution was decanted and centrifuged at 20,000 g for 20 min. The 20,000 g pellet, designated subfraction HM, was washed once and resuspended in the appropriate buffer for assays (usually in buffer B). The 20,000 g supernatant solution was then fractionated for spearation of rough and smooth microsomes; the discontinuous sucrose-gradient method of Dallner was used (13) by making

[2] All g values are for the tube bottom.

the solution 15 mM in CsCl and layering it in 10-ml aliquots on 2.5 ml of buffer C in cellulose nitrate tubes of the SW 41 rotor. Upon centrifugation at 272,000 *g* for 6 hr, the rough microsomes formed a loose pellet at the tube bottom, while the smooth microsomes formed a fluffy band at the interface. 8 ml of the supernatant solution were pipetted from each tube. The smooth microsomes were then removed by pipette with 2 ml of supernatant solution and about 0.2 ml of buffer C. After the removal of an additional milliliter of clear buffer C the remaining rough microsomes in the dense sucrose buffer were removed. The rough microsomes were diluted three times and the smooth microsomes five times with buffer A. After centrifugation of both fractions at 358,000 *g* for 60 min, the resulting pellets were washed once with buffer A and resuspended in 3–5 ml of buffers appropriate for specific enzyme assays.

Electron Microscopy

Portions of the fractions, in buffer A, were centrifuged at 201,000 *g* for 60 min. The pellets were fixed in collidine-buffered 1% osmium tetroxide for 45–60 min. The fixed pellets were dehydrated in alcohol and embedded in Epon. Thin sections were routinely cut in a vertical direction from the center of the pellet, doubly stained with uranyl acetate and lead citrate, and examined in a RCA EMU-3F electron microscope.

Analytical Methods

For the determination of protein, preparations were precipitated with 10% trichloroacetic acid, washed twice with 5% trichloroacetic acid, and dissolved 1 N KOH. Protein was determined by the biuret (17) or Lowry et al. (18) methods.

Determinations of RNA were carried out after the removal of interfering mucopolysaccharides. Preparations were digested with 2000 IU of testicular hyaluronidase in sodium acetate, 0.1 M; NaCl, 0.15 M; pH 5.0, at 37°C for 7 hr. Cold trichloroacetic acid was added to a final concentration of 10%. Resulting pellets were washed twice with cold 5% trichloracetic acid, and the RNA was extracted in 5% trichloroacetic acid at 90°C for 15 min. RNA content was determined by orcinol-pentose analyses (19) with yeast RNA as standard.

Uronic acid analyses could not be carried out directly because of interference by RNA. Samples were therefore digested with papain for 16 hr, after which the enzyme was denatured at 100°C for 5 min. The mixture was then dialyzed against several changes of 0.01 M sodium phosphate, pH 7.4, followed by digestion with ribonuclease in phosphate buffer, pH 7.4, with 0.005 M $MgCl_2$. The resulting solution was placed on a column of Sephadex G25, 10 × 500 mm, and eluted with 0.2 M NaCl. Fractions collected were analyzed by the carbazole method (20). The chondroitin sulfate was present in the excluded volume of the column.

To determine phospholipid content, we extracted trichloroacetic acid precipitates twice at room temperature for 30 min with chloroform:methanol (2:1, v/v). The extracts were washed according to the procedure of Folch et al. (21), and phosphorus was determined by the method of Fiske and SubbaRow (22). Phospholipid was estimated by multiplying the phosphorus content by 25.

Radioactivity determinations were carried out in a Packard Tri-Carb model 3375 scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) in the counting system previously described (3). Automatic external standardization was used for determination of efficiencies of counting and absolute activities.

Enzymic Assays

XYLOSYLTRANSFERASE AND GALACTOSYLTRANSFEASE: The subcellular fractions were suspended in buffer B, but with 0.005 M $MnCl_2$ and at pH 6.5. For the study of xylose incorporation, the incubation mixture contained 8.43 mμmoles (0.243 μc) of UDP-Xyl-^{14}C with 1–2 mg of enzyme protein in 1.0 ml. In the study of galactose incorporation, the reaction mixture contained 10.0 mμmoles (0.182 μc) of UDP-Gal-^{14}C in addition to the enzyme preparation. After incubation at 37°C for 60 min, the reaction was stopped by precipitation of the protein with an equal volume of 10% trichloracetic acid. The precipitates were washed twice with cold 5% trichloroacetic acid and extracted twice with chloroform:methanol (2:1, v/v) at room temperature for 30 min. The residue was dried and dissolved in 1 N KOH, and aliquots were taken for counting and protein determinations.

CHONDROITIN SULFATE POLYMERASE SYSTEM: The polymerase activity is defined by the enzymatic addition of hexosamine and uronic acid residues. It is not yet clear whether one or two enzymes are involved. Polymerase activity was determined by two methods. The first involved the measurement of incorporation of radioactivity into large molecular weight material in the presence of both UDP-GalNAc and UDP-GlcUA. The second method involved the addition of single sugar residues to oligosaccharides as described by Telser et al. (4). In this method *N*-acetylgalactosamine was added to the nonreducing uronic acid of a hexasaccharide to form a heptasaccharide, and glucuronic acid was added to the nonreducing *N*-acetylgalactosamine of a pentasaccharide to form a hexasaccharide.

For the measurement of synthesis of large molecular weight material, 1–2 mg of enzyme protein was incubated with 0.25 μmole of UDP-GalNAc-^{3}H (1.0 μc) and 0.25 μmole of UDP-GlcUA in a total volume

of 1.0 ml. After incubation at 37°C for 60 min, the reaction was stopped by heating to 100°C for 3 min. 2 mg of carrier CS were added, and the CS was isolated as follows. The mixtures were digested with papain at 60°C overnight, boiled, and centrifuged at 10,000 *g* for 10 min; the supernatant solutions were dialyzed exhaustively against water. From the solutions, made 0.03 M in NaCl, CS was precipitated by adding 5% CPC dropwise. The precipitates were washed twice with 0.1% CPC in 0.03 M NaCl, dissolved in 2.0 M $CaCl_2$, and reprecipitated with 9 volumes of ethanol:ether (2:1, v/v). The residue was dried with ether and dissolved in water, and samples were taken for counting and uronic acid determinations.

The oligosaccharide assay method (4) was modified to give a rapid and reproducible assay for CS polymerizing activity. It is based on the use of a CPC-cellulose column (23) as indicated below. For the measurement of heptasaccharide formation, the subfractions, in buffer B, were incubated with 150 μg of CS-4 hexa and 24 mμmoles (0.5 μc) of UDP-GalNAc-^{3}H in a total volume of 0.25 ml. In the measurement of the hexasaccharide formation, the incubation included 200 μg of CS-6 penta and 38 mμmoles (0.184 μc) of UDP-GlcUA-^{14}C. The mixtures were incubated at 37°C for 60 min, and the reactions were stopped by heating them to 100°C for 3 min. 1 ml of water was added, the tubes were centrifuged, and the supernatant solutions were applied to CPC-cellulose columns for analyses.

The CPC-cellulose columns were prepared in disposable pipettes by adding cellulose powder in water to form a column, 5 × 50 mm. The cellulose was saturated with 0.25% CPC in water. After addition of the sample, the columns were washed with 2 ml of water followed by 10 ml of 0.03 M NaCl for removal of unreacted, labeled nucleotide sugar. The oligosaccharide was eluted with 3 ml of 0.3 M NaCl. Occasionally the column was eluted with 1.5 N $MgCl_2$ for removal of any high molecular weight CS. Aliquots of the elutions were taken for radioactivity and uronic acid determinations. Routinely, the last milliliter of the 0.03 M NaCl eluent was counted to insure that precursor had been removed.

SULFOTRANSFERASE ASSAY: Fractions for sulfotransferase assay were prepared and assayed in the following buffer: sucrose, 0.2 M; MES, 0.05 M (pH 7.0); KCl, 0.05 M; $MnCl_2$, 0.003 M; $MgCl_2$, 0.005 M; cysteine, 0.006 M. Fractions prepared in buffer A showed similar distribution but lower levels of activity. The preparations were incubated with 24 mμmoles (0.4 μc) of PAPS-^{35}S at 37°C for 60 min. Reactions were stopped by heating at 100°C for 3 min, 2 mg of carrier CS were added, and CS was isolated as described above.

RESULTS

Morphological and Chemical Characteristics of Microsomal Subfractions

The currently accepted criteria for the identification of isolated rough and smooth microsomes are based on electron microscopic verification of morphology and on chemical composition including RNA-to-protein and phospholipid-to-protein ratios. The composition of the subfractions is similar to that of subfractions of liver (13), pancreas (7), and brain (24).

Electron micrographs of the chondrocyte microsomal subfractions show intact vesicles derived from healed fragments of endoplasmic reticulum and Golgi complex. Fig. 2 *a* shows the rough subfraction to be made up of membrane-bounded vesicles with attached ribosomes. Most of the vesicles contain an electron-opaque material. In contrast, Fig. 2 *b* indicates that the smooth fraction is composed of smooth-surfaced, membrane-bounded vesicles with few ribosomes. These vesicles also contain some electron-opaque material. The 20,000 *g* pellet, fraction HM, is shown in Fig. 2 *c*. This fraction is more heterogeneous and consists of much larger vesicles, usually with dense contents. The majority of the vesicles are associated with ribosomes. This fraction appears to contain larger fragments of endoplasmic reticulum than the purified rough and smooth preparations.

The chemical composition of the subfractions obtained by the discontinuous gradient method is given in Table I. The protein content is for preparations obtained by the fractionation of pools of epiphyses from 54 embryos. The observed RNA-to-protein and phospholipid-to-protein ratios are consistent with published data for similar fractions from other tissues. The uronic acid-to-protein ratios reflect the relative CS content of the fractions. Analyses of the smooth microsomes yield a uronic acid-to-protein ratio about four times that of the rough microsomes. The RNA-to-protein ratio of the HM fraction is lower than might be expected on the basis of the electron microscopic appearance; this may be due to the relatively high nonmembrane protein content of that fraction.

Separations were also carried out with the use of the linear density-gradient method of Jamieson and Palade (7). In their morphology, chemical composition, and enzymic activities the rough

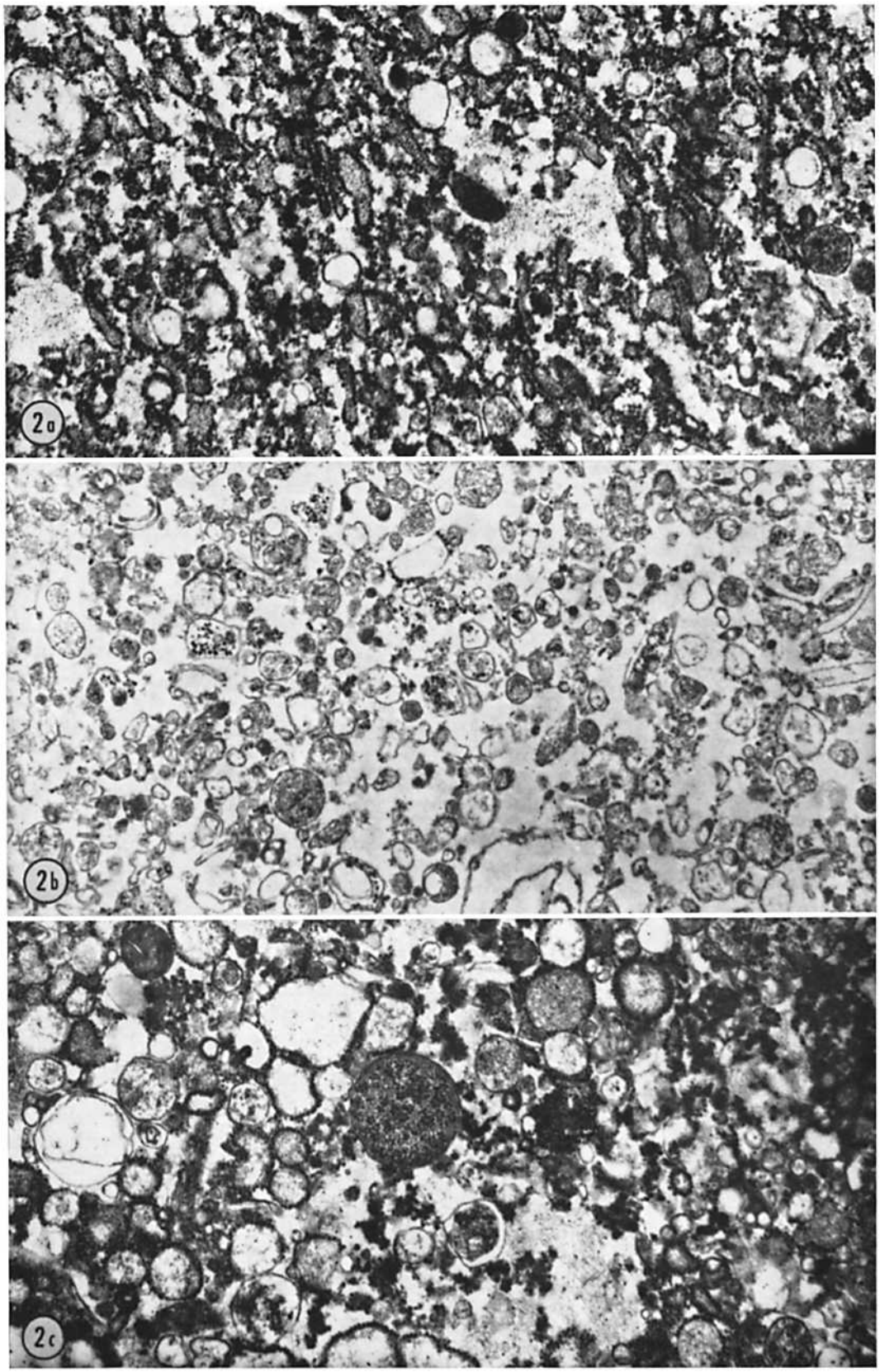

FIGURE 2 *a–c* Micrographs of sections from the middle of the rough (Fig. 2 *a*) and smooth (Fig. 2 *b*) microsomal pellets, and of a pellet of the HM fraction (Fig. 2 *c*). × 28,000.

and smooth subfractions were similar to those prepared by the discontinuous gradient method, but there was a greater proportion of rough microsomes in the linear gradient-separated material. Since the linear gradient method was somewhat more difficult for routine use, the discontinuous gradient method was used for obtaining material for enzymic studies. As a test of the purity of the subfractions prepared by the discontinuous gradient method, portions were layered on a linear sucrose-density gradient (1.0–2.0 M). The gradients were centrifuged to form density equilibrium bands, and fractions were collected dropwise and examined for ultraviolet absorbancy. The rough subfraction formed a single sharp band three-quarters of the way down the gradient at a modal density of 1.290, while the smooth microsomes formed a broader band near the top of the gradient at a modal density of 1.175. The HM fraction was also a single band in the region of the rough microsomes at a modal density of 1.285.

The morphological and chemical compositions of the subfractions thus show that the method of separation of rough from smooth microsomes can be successfully applied to cartilage.

TABLE I

*Composition of Subcellular Fractions**

Fraction	Protein‡	RNA / protein	Uronic acid / Protein	Phospholipid / Protein
	mg			
Rough	1.67	0.252	0.025	0.27
Smooth	3.60	0.077	0.103	0.59
HM	4.20	0.081	0.027	0.19

* Discontinuous gradient.

‡ After cold trichloroacetic acid and chloroform-methanol extraction.

Distribution of Enzymes Involved in Chondroitin Sulfate Biosynthesis

FORMATION OF THE PROTEIN-POLYSACCHARIDE LINKAGE REGION: Chondroitin sulfate chain-initiation involves the formation of a xylosylserine O-glycosidic bond by the transfer of xylose from UDP-xylose to hydroxyl groups of serine of the protein core (2). The submicrosomal distribution of the enzymic activity involved in the transfer of xylose-^{14}C into trichloroacetic-acid precipitable material is given in Table II. Both the total activity and specific activity are much higher in the rough subfraction than in the smooth. The HM fraction also contains considerable enzyme activity but the specific activity based on protein content is lower than in the rough fraction.

Next in the linkage region sequence are two galactose residues. The transfer of galactose-^{14}C into trichloroacetic acid–precipitable material is also shown in Table II. In this case also, the enzyme activity of the rough fraction is greater than that of the smooth. The HM fraction again shows high total activity but lower specific activity.

It should be noted that the xylosyltransferase and galactosyltransferase assays contain two

TABLE II

Xylosyltransferase and Galactosyltransferase Assays of Submicrosomal Fractions

Precursor	Fraction	Incubation time	Total activity	Specific activity
		min	*dpm*	*dpm/mg protein*
UDP-xylose-^{14}C	Rough	0	82	86
		60	4390	5000
	Smooth	0	66	54
		60	690	585
	HM	0	48	22
		60	5100	2270
UDP-galactose-^{14}C	Rough	0	105	110
		60	7650	8350
	Smooth	0	89	63
		60	710	505
	HM	0	100	43
		60	10150	5040

variables. The first is the concentration of enzyme; the second is the availability of acceptor protein. It is not known which variable is limiting. It can be concluded, in any case, that the total activity for addition of the linkage region sugars, xylose and galactose, to protein acceptor is greater in the rough than the smooth microsomes.

CHONDROITIN SULFATE POLYMERASE ACTIVITY:

The polymerase activity of the microsomal subfractions to form chondroitin sulfate chains from the uridine diphosphate sugars is given in Table III. In this case the specific activity is approximately equal in the smooth and rough fractions.

It is to be noted that omission of UDP-GlcUA decreases incorporation of isotope from UDP-GalNAc-^{3}H in the rough, smooth, and HM fractions, in accord with previous observations (3). This suggests that elongation by more than one sugar residue is being measured when both nucleotides are present. In contrast, the 314,000 *g* supernatant fractions (Table III) showed no depression of activity when UDP-GlcUA was omitted. In a similar manner the omission of UDP-GalNAc, when incorporation of radioactivity from UDP-GlcUA-^{14}C was measured showed the same phenomenon. The significance of these observations is not clear, but they indicate a failure of chain elongation by more than one sugar residue with this supernatant preparation.

TABLE III

Chondroitin Sulfate Polymerase Activity of Submicrosomal Fractions

Fraction	Addition* UDP-GlcUA	Incubation time	Total activity	Specific activity
		min	*dpm/mg UA*	*dpm/mg UA/mg protein*
Rough	+	0	52	68
	+	60	3000	3850
	−	60	1600	2050
Smooth	+	0	79	62
	+	60	5850	4560
	−	60	1650	1290
HM	+	0	178	125
	+	60	8680	6120
	−	60	1040	730
Supernatant fraction	+	0	23	13
	+	60	1770	985
	−	60	1630	905

* All incubations contained UDP-GalNAc-^{3}H

As is the case with xylose and galactose incorporation, the polymerization assay with both nucleotide sugars is dependent on the concentration of endogenous acceptor as well as the concentration of polymerase.

The measurement of CS polymerase activity with oligosaccharide acceptors obviates the problem of endogenous acceptors. Table IV gives the results of experiments in which the formation of hepta- and hexasaccharides is measured with the oligosaccharide assay method described above. When the oligosaccharide acceptor was omitted from the reaction mixture, no formation of a radioactive compound was observed. Thus the activity measured is a function of enzyme concentration rather than of endogenous acceptor concentration.

It is to be noted that CS-6 penta served as the acceptor for GlcUA in studies reported in Table IV. Previous reports indicated that the pentasaccharide from CS-4-SO_4 does not act as an acceptor, although the desulfated compound does serve as acceptor. The desulfated compound was not useful for these studies since it is not retained by the CPC-cellulose columns.

The results of the oligosaccharide acceptor assay resemble those of the measurement of formation of large molecular weight material. With the hexasaccharide as acceptor for labeled GalNAc-^{3}H, the specific activity is about the same in the rough and smooth fractions, but the HM fraction has a greater specific activity than do the other two fractions. For the pentasaccharide assay the rough subfraction is somewhat higher. The explanation of this discrepancy is not clear.

In any case, it is apparent from both methods of assay for condroitin sulfate polymerase that both the rough and smooth microsomal subfractions display approximately the same specific activity.

DISTRIBUTION OF SULFOTRANSFERASE IN SUBMICROSOMAL FRACTIONS: The transfer of sulfate from PAPS to CS is also carried out by the microsomal fraction. In an earlier report from this laboratory, sulfation with $^{35}SO_4$ was found in the supernatant fraction. Subsequent studies[3] showed that the enzymes required for activation are in this supernatant fraction, but the sulfotransferase activity for transfer of SO_4 from PAPS to polysaccharide is primarily concentrated in the particulate fraction. The distribution of this activity

[3] Robinson, H. C., A. Telser, and A. Dorfman. Unpublished observations.

TABLE IV

Oligosaccharide-Polymerase Activity of Submicrosomal Fractions

Nucleotide sugar	Fraction	Incubation time	Acceptor	Total activity	Specific activity
		min		*dpm*	*dpm/μg UA/ mg protein*
			CS-4 hexa		
UDP-GalNAc-^{3}H	Rough	0	+	0	0
		60	+	15500	3800
		60	−	575	—
	Smooth	0	+	410	46
		60	+	23300	2620
		60	−	400	—
	HM	0	+	325	24
		60	+	65500	4840
		60	−	650	—
			CS-6 penta		
UDP-GlcUA-^{14}C	Rough	0	+	116	140
		60	+	5600	1020
		60	−	270	—
	Smooth	0	+	320	27
		60	+	3880	370
		60	−	300	—
	HM	0	+	410	28
		60	+	9640	510
		60	−	580	—

TABLE V

Sulfotransferase Activity of Subcellular Fractions

Fraction	Incubation time	Specific activity
	min	*dpm/mg UA/mg protein*
Rough	0	840
	60	16400
Smooth	0	2610
	60	81500
HM	0	229
	60	35900

in microsomal subfractions is given in Table V. It was found that the presence of magnesium during the preparation as well as during assay enhanced the sulfotransferase activity. The specific activity of the smooth subfraction is four to five times that of the rough fraction and about twice that of the HM fraction. This assay, however, is dependent on endogenous acceptor. Although the exact nature of the endogenous acceptor is unknown, the smooth subfraction contains about four times as much uronic acid–containing material and presumably four times as much endogenous acceptor as does the rough subfraction.

DISCUSSION

That proteins are synthesized at the rough endoplasmic reticulum, transferred to the smooth reticulum or Golgi complex, and then exported from the cell in such tissues as liver and pancreas is supported by considerable evidence (6, 7). However, the patterns of synthesis and transport may vary. Substances for export from the cell may be modified in various ways. The simplest way, perhaps, is aggregation and condensation to form secretory granules. Thus, for pancreatic enzymes, the zymogens are accumulated and condensed, and the packets are separated from the Golgi complex (25). In the case of liver glycoproteins, the proteins are modified by addition of short carbohydrate units. The hexosamine is believed to be added, at least in part, at the ribosomes (12), and perhaps additional sugars are added as the proteins traverse the cisternal spaces. Another interesting example of modification prior to export is that of protocollagen which must be hydroxylated before export (26). Chondromucoprotein may be considered an extreme case of modification of a protein which involves the addition of extended polysaccharide chains. These chains in turn are further modified by sulfation.

The data presented in this paper indicate that the enzymic activities involved in CS synthesis exist in the rough and the smooth microsomes. As noted in Results, only in the case of the assay for polymerase can the concentration of active enzyme be considered rate limiting. In all other assays performed, the observed activity may be limited by the concentration of either active enzyme or acceptor or both. Therefore it cannot be concluded from our data whether there is a quantitative difference in enzyme concentration between the rough and smooth microsomes.

Since the protein must be synthesized at the rough endoplasmic reticulum, the rough microsomes might be expected to contain polypeptide chains having free serine for addition of xylose and subsequently galactose residues. The small amount of xylosyltransferase and galactosyltransferase activities found in the smooth fraction may be due to contamination with rough fraction. After formation of the linkage region, one must envision a continuing process of the addition of alternating glucuronic acid and *N*-acetylgalactosamine residues, perhaps even before the peptide chain is completed. The enzymes necessary for CS chain-initiation, chain elongation, and, to some extent, sulfation are present in the rough microsomal fraction. The smooth microsomes contain the enzymes involved in chain extension and sulfation.

The results of these experiments are at some variance with radioautographic studies with $^{35}SO_4$ (8, 9) and triated galactose (10). These studies implied that the Golgi complex was the major, if not the only, site of CS synthesis. However, the lack of resolution in thick sections with relatively high energy $^{35}SO_4$ and the absence of electron microscopic evidence in the tritiated galactose studies should be taken into account. The appearance of radioactivity in the Golgi apparatus may simply reflect a concentration of product in this area. It is possible, however, that in vivo the major part of the polymerization does occur in the Golgi complex because of the increasing concentration of endogenous acceptor in this region. Our studies, however, do show that both enzymes and acceptor are present in rough microsomes corresponding to the rough endoplasmic reticulum.

For a more complete understanding of the mechanism of formation of complex macromolecules, such as chondromucoprotein, which are destined for export, a number of factors must be considered: (*a*) the localization of synthesis of the core protein; (*b*) the site and time of addition of the initial carbohydrate unit; (*c*) the site and the spatial organization of the enzymes responsible for chain elongation and sulfation; (*d*) the mechanisms reponsible for chain termination; (*e*) the mechanism responsible for the release of the completed product from the cell. It seems reasonable to assume that the core protein is synthesized at the ribosome by a conventional pattern of protein synthesis. It should be noted that in differentiated connective tissue cells, which are making large amounts of matrix, the ribosomes are organized on a membrane system to form a extensive rough endoplasmic reticulum (27). Two questions regarding the core protein at this stage may be raised. Is the first carbohydrate residue added to the protein before the polypeptide chain is completed? After the protein is completed (on the ribosome), is the product transferred into some intercisternal space or does it become part of a membrane system before completion of the polysaccharide chain? As for the first problem, studies of glycoprotein synthesis indicate that part of the hexosamine is added to the protein while the latter is still attached to the ribosome. Preliminary results in our laboratory suggest that a similar phenomenon occurs for the addition of xylose. Whether the protein is complete when this happens is not yet clear.

On the basis of our studies and other available information, we may interpret our data as follows. The synthesis of chondromucoprotein is initiated by formation of the core protein at the ribosome. The linkage sugars are added primarily at the rough endoplasmic reticulum, and the molecule is elongated and completed as it proceeds through the smooth endoplasmic reticulum to final secretion. The addition of *N*-acetylgalactosamine and glucuronic acid units occurs in both the rough and smooth fractions. The finding of higher activity for xylose and galactose incorporation in the rough endoplasmic reticulum may primarily result from a higher concentration of acceptor in this region, while the higher incorporation of sulfate in the smooth endoplasmic reticulum results from the higher concentration of appropriate polysaccharide acceptor. The latter possibility is attested by the higher uronic acid concentration in the smooth microsomes.

Before further considering the final fate of the product, we may now turn attention to the enzymes responsible for the completion of the carbo-

hydrate chains. Irrespective of the localization demonstrated in our studies, it is quite clear that the various enzymes involved are closely associated with membranes. It seems logical to assume that this complement of enzymes is synthesized at ribosomal sites, and that the enzymes move through the cells from the rough endoplasmic reticulum to the smooth. Evidence for such a flow of both membranes and certain enzymes has appeared (28, 29).

The localization on membranes of enzymes involved in the synthesis of complex macromolecules has been widely recognized. Although hypothetical, it is reasonable to assume that the specific spatial arrangement of enzymes active in a series of metabolic reactions may be highly advantageous from a kinetic point of view. If all the reactions were to proceed randomly in free solution, each product would have to achieve relatively high concentrations before it could saturate the next enzyme in the biosynthetic pathway. The data in Table III may indeed reflect the inefficiency of such a system. Although the 314,000 *g* supernatant fraction is able to transfer both *N*-acetylgalactosamine and uronic acid from the requisite nucleotides, the presence of the alternate nucleotide sugar did not result in stimulation of incorporation of radioactivity. These results suggest that in this soluble fraction only one sugar residue is added to each acceptor molecule, no further chain elongation occurring.

If then, as apparently is true, the enzymes are inserted into a membrane as they are synthesized at the ribosomal site, the progression of biosynthetic reactions may be promoted by the spatial relationships of the enzymes. One might speculate further and suggest that the cistrons for enzymes responsible for sequential addition of monosaccharides may be sequentially located in the chromosome so that, as messenger RNA is read by the ribosome, the enzymes manufactured are inserted into the membrane in an orderly fashion. By this mechanism the RNA template may actually be producing a secondary type of template for orderly sequence of growth of a polysaccharide chain. The result of this process would be the appearance on both the rough and smooth microsomal membranes of the sequence of enzymes involved in the synthesis of chondromucoprotein. The detectable functioning of these multienzyme units would depend on the availability of acceptor substrates which in turn would represent a stage in the completion of the polysaccharide chains. We may consider this assembly of enzymes as a factory assembly line which is renewed at the ribosomes. Similarly, the acceptor protein is formed at the ribosome and probably inserted into a membrane.

One may then consider the entire process as follows. An assembly line of enzymes oriented in an orderly fashion is formed at the ribosome and passes slowly (half-life time of the order of magnitude of days) along a path toward the Golgi apparatus. Along this path is travelling a product which is being fabricated by this enzymatic machinery. The product travels much more rapidly (half-life time probably of the order of magnitude of minutes). Whether the product is in solution in the cisternae of the endoplasmic reticulum or is attached to a separate membrane with more rapid transport time is not clear. The completed product accumulates in vesicles and is finally exported by reverse pinocytosis (27). The factors that control chain termination, release from membrane, and finally extrusion are unknown.

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