Localization of Proteoglycan Core Protein in Subcellular Fractions Isolated from Rat Chondrosarcoma Chondrocytes*

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Chondrocytes from the Swarm rat chondrosarcoma were pulse-labeled with [3H]serine for 30 min and chased, in the presence of cycloheximide, for times up to 300 min. The movement of newly synthesized core protein precursor of the proteoglycan through elements of the endoplasmic reticulum and Golgi complex was examined. Rough and smooth microsome fractions were obtained by centrifuging postmitochondrial supernatants from cell homogenates on discontinuous sucrose gradients. The core protein precursor was identified in subcellular fractions by (a) immunoprecipitation with an antisera directed against the hyaluronate binding region of the core protein and the link protein and (b) its size on polycrylamide gels. Labeled core protein precursor decreased from the microsomes with a t1/2 of 60 ± 9 min, nearly the same as for the appearance of label in completed proteoglycan monomer (t1/2 = 58 ± 13 min), consistent with a precursor-product relationship. After correcting for incomplete recovery of the core protein precursor in the microsomal fractions and for cross-contamination of the smooth microsomes by elements of rough endoplasmic reticulum, the redistribution of core protein precursor and completed proteoglycan in the intracellular compartments and of labeled extracellular proteoglycan were fit to a three-compartment model. A t1/2 of 98 ± 7 min for the loss of core protein precursor from the rough microsomes and a t1/2 = 10 ± 4 min for the completed proteoglycan in the intracellular compartment (Golgi and secretory vesicles) was obtained. The data indicate that at least 70% of the intracellular transit time for the core protein precursor is spent in the rough endoplasmic reticulum. The addition of glycosaminoglycan chains followed by secretion from the cell occurs relatively rapidly, occupying less than 30% of the total intracellular dwell time.

Biosynthesis of the cartilage proteoglycan with a core protein of molecular weight between 2 and 4 x 10^4 (1–3) is initiated with the synthesis of a core protein precursor with an apparent molecular weight of 3.5–4 x 10^4 (4, 5). In subsequent steps, N-linked and O-linked oligosaccharides as well as the larger chondroitin sulfate and keratan sulfate glycosaminoglycans are added on the core protein (6–9). Experiments using chondrocytes derived from the Swarm rat chondrosarcoma have identified a pool of core protein precursor which has a half-time of approximately 90 min before chondroitin sulfate is added (5, 10, 11). Based on what occurs in the biosynthesis of other glycoproteins (12), it is likely that N-linked, high mannose oligosaccharides are added to nascent or newly synthesized core protein precursor in the rough endoplasmic reticulum. Several investigations indicate that completion of the proteoglycan structure by adding chondroitin sulfate chains and O-linked oligosaccharides occurs in the Golgi complex. For example, Silbert and Freilich (13) prepared an enriched Golgi fraction from fibroblasts and showed that [35S]sulfate incorporation into glycosaminoglycan was predominantly localized in this fraction. Furthermore, electron microscopy and radioautography of chondrocytes pulse-labeled with [3H]serine showed that the Golgi complex is the initial site for incorporation of this precursor into macromolecules (14–17). In a previous report (18), evidence was presented indicating that O-linked oligosaccharides are added onto the core protein at the same time chondroitin sulfate is added and therefore occurs at the same intracellular site.

Sulfation, probably the last step in completing the proteoglycan, occurs rapidly once initiated. Kimura et al. (10), using rat chondrosarcoma chondrocytes, have shown that the size distribution of proteoglycans labeled for only 2 min with [35S]sulfate is indistinguishable from that found for proteoglycans recovered after a 15-min chase when the majority of the molecules have been secreted. Since no partially completed proteoglycan molecules could be detected immediately after the short pulse, addition of chondroitin sulfate chains must be a very rapid step. The newly completed, [35S]sulfate-labeled proteoglycans are subsequently secreted from the cell with a t1/2 of about 5 min (19). In sum, these data suggest that completion of the proteoglycan in the Golgi complex and subsequent secretion occur rapidly compared to the intracellular half-time of the core protein precursor. Thus, the rate-limiting step in the biosynthetic process may be either the transport of the core protein precursor from the rough endoplasmic reticulum to the Golgi or the addition of glycosaminoglycan chains and O-linked oligosaccharides in the Golgi.

Techniques in which cells are fractionated into subcellular components such as rough and smooth microsomes used in combination with radioactive pulse-chase techniques have been used to follow the synthesis, assembly, and secretion of a variety of macromolecules (20–28, among others). For the results described in this paper, a subcellular fractionation procedure was used in combination with kinetic experiments to show that the core protein precursor is located in the rough endoplasmic reticulum for most of its intracellular dwell time.

EXPERIMENTAL PROCEDURES

Materials—[G-3H]Serine (16.8 Ci/mmol), [35S]sulfate (680–800 mCi/mmol), and [5-3H]uridine (25 Ci/mmol) were purchased from...
New England Nuclear and [2-3H]mannose (16.1 Ci/mmol) and [1-3H]glucose (6.7 Ci/mmol) from Amersham. Zwittergent-3-12 was purchased from Calbiochem, and BSA was from Boehringer Mannheim, and 2-naphthol-4-sulfonic acid from Fisher. [3H]Serine-labeled A1 and A1-D1 proteoglycan samples were prepared as described elsewhere (29).

Cell Culture and Labeling—Cells from the Swarm rat chondrosarcoma were grown in monolayer culture with continuous treatment with trypsin and collagenase as described in Kimura et al. (19). The chondrocytes were stored at 4 °C overnight in Dulbecco’s modified Eagle’s medium with 4.5 g/liter of glucose supplemented with 20% fetal calf serum, 10 mM Hepes, pH 7.2, plus antibiotics (20). Cells were plated at a density of 40 × 10^4 cells/100 mm culture plate in 10 ml of medium and allowed to attach for 2–3 h in a 5% CO2, 95% air atmosphere at 37 °C. Short culture times were necessary to prevent the formation of an appreciable amount of extracellular matrix, which increased the resistance of the chondrocytes to subsequent breakage with homogenization. Cells were labeled as follows for the analyses of precursor incorporation: (a) 25–50 μCi/ml of [3H]serine and 100 μCi/ml of [35S]sulfate in 2 ml of culture medium prepared without serine and without sodium sulfate for 10 min and 2 min, respectively; (b) 100 μCi/ml of [3H]uridine in 5 ml of medium for 180 min; (c) 50 μCi/ml of [3H]mannose in 10 ml of medium for 50 μCi/ml of [3H]serine in 10 ml of culture medium prepared without glucose and supplemented with 15 mM pyruvate for 270 min. For immunoprecipitation of labeled molecules in microsomes, cultures were labeled with 100 μCi/ml of [3H]serine for 180 min. Incorporation was halted by the addition of cycloheximide, followed by chilling on ice. Culture medium from each culture to be fractionated was neutralized with a saturated Tris-base solution. The samples were transferred to glass-Teflon homogenizers. The cells were broken by three cycles of five strokes with the pestle motor driven at 3500 rpm. The homogenate was centrifuged at 5000 rpm in a Beckman JA-20 rotor for 7 min. The resulting postmitochondrial supernatant (500,000 g, 15 min) was layered on a discontinuous sucrose gradient consisting of 1.7 ml of 2.0 M sucrose, 4.3 ml of 1.24 M sucrose, and 2.0 ml of 0.5 M sucrose, each fractionated by puncturing the bottoms of the cellulose nitrate tubes with a 22-gauge needle and collecting 16 fractions of 1 ml each. After analyses, fractions 2 and 3 were combined to give the rough microsome fraction and fractions 6 and 7 combined to give the smooth microsome fraction (see Fig. 1 below). Alternatively, for some experiments, the subcellular fractions, which appeared as turbid bands, were removed directly from the sucrose interfaces by aspiration with a large bore needle.

Analyses of Gradient Fractions—Macromolecular radioactivity of fractions was determined by making aliquots of each fraction 15% in trichloroacetic acid, pelleting by centrifugation, and washing the resulting precipitate three times with cold 10% trichloroacetic acid. Each final pellet was solubilized in 0.5 ml of 4 M guanidine HCl and neutralized with a saturated Tris-base solution. The samples were transferred to glass scintillation vials to which 0.5 ml of 70% ethanol and 10 ml of Hydrofluor were added before counting in a Beckman LS9000 scintillation counter. Analysis of fractions before and after trichloroacetic acid precipitation on Pharmacia PD-10 columns eluted with 4 M guanidine HCl indicated that the amount of unincorporated acid-labile 3H activity in fractions 1–9 was less than 5% even before trichloroacetic acid precipitation, while that for fractions 10–16 was less than 15% (see Fig. 1 below). Protein contents of the trichloroacetic acid precipitates were determined by the Lowry method (31). Assays for glucose-6-phosphatase and 5’-nucleotidase (5’-adenosine monophosphatase) were done as described elsewhere (32). For each assay, 50–60 μl aliquots of the gradient fractions were diluted with 0.45 ml of the respective reaction mixtures and were incubated at 37 °C for 30 min (in the 5’-nucleotidase assay) and for 6 h (in the glucose-6-phosphatase assay). Both reactions were linear for their respective reaction times. The data are presented as micromoles of inorganic phosphate released/min/mg of protein.

Quantitation of Core Protein—Fractions isolated during subcellular fractionation were precipitated with 3 volumes of 95% ethanol at −20 °C overnight. Precipitates were washed 2–3 times with 70% ethanol, 1% (w/v) sodium acetate and solubilized in SDS-sample buffer. Aliquots were fractionated on PD-10 columns to determine total macromolecular radioactivity in the samples. Other aliquots were fractionated by electrophoresis on SDS-polyacrylamide gel and radioactivity was determined in slices, as previously described (5). The per cent of the total activity on the gel which was present as core protein precursor was multiplied by the total "H activity in the microsomal fraction to give the total "H activity present as core protein precursor.

Pulse-Chase Experiment—Six chondrocyte cultures were pulse-labeled with 250 μCi/ml of [3H]serine in 1.5 ml of serine-free medium for 30 min. The pulse was ended by making the medium 100 μg/ml of cycloheximide, followed by two washes with balanced salt solution containing 100 μg/ml of cycloheximide (10). The 0-min chase culture was immediately chilled on ice and processed for fractionation. Other cultures were chased in complete medium containing 100 μg/ml of cycloheximide for 15 min, 45 min, 90 min, 180 min, and 300 min. After fractionation, the rough and smooth microsome fractions were precipitated with 3 volumes of 95% ethanol and washed as described above. Five-sixths of each ethanol-precipitated microsomal fraction was pelleted, solubilized in 0.5 ml of SDS-sample buffer, and an aliquot fractionated on SDS-polyacrylamide gel and the data are presented as micromoles of inorganic phosphate released/min/mg of protein.

The total amount of radiolabeled core protein precursor in each microsomal fraction was determined as described above.

The remaining one-sixth of each ethanol-precipitated fraction was pelleted and solubilized in 4 M guanidine HCl, 4% (w/v) Zwittergent and a D1-proteoglycan fraction prepared by CsCl density gradient centrifugation (see below). The proportion of the total gradient activity in the D1 fraction was multiplied by the total activity in the microsomal fraction to give the "H activity in completed monomer for each microsomal fraction. The D1-proteoglycan fractions were prepared from the medium from each culture by a dissociative CsCl-density gradient centrifugation to give the total "H activity in secreted material.

Kinetic data from the above experiment were analyzed assuming a three-compartment system with unidirectional flow of label between compartments. Cycloheximide was used to reduce the generation of labeled material in the endoplasmic reticulum essentially to zero (5, 10). Therefore, the kinetic data measured the redistribution of labeled material in the culture system from the rough endoplasmic reticulum to the Golgi fraction and then out of the cell. Previous results have suggested that the conversion of core protein to complete proteoglycan in these cultures follows nearly first order kinetics (5), in which the rate of loss of label from a compartment is proportional to the amount remaining in that compartment at a given time. Thus, for Rough = K1 → smooth → K2 → extracellular

where K1 and K2 are, respectively, rate constants for the loss of label from the rough and smooth microsome compartments.

\[
\begin{align*}
\frac{dR}{dt} &= -K1 \cdot R(t) \\
\frac{dS}{dt} &= K1 \cdot R(t) - K2 \cdot S(t) \\
\frac{dE}{dt} &= K2 \cdot S(t)
\end{align*}
\]

where the K = ln 2/1/2 and RM, SM, and EX are the concentrations of label in the rough, smooth, and extracellular compartments, respectively.

Corrections were applied to the experimental data as described under "Results" and the relative amounts of label in each compartment at the six time points were used to generate curves fitted to the
above system of equations. Curve fitting and the calculation of half-time values were done using the MLAB (modeling laboratory) program running on the DecSystem-10 at the National Institutes of Health.

**Immunoprecipitation**—Antiserum R98 was prepared in rabbits immunized with a ternary complex of hyaluronic acid-binding region/hyaluronic acid/link protein and contains antibodies to both the hyaluronic acid-binding region of the core protein and the link protein (5, 33). Immunoprecipitation of antigens from subcellular fractions using the indirect antibody technique was done as described previously (5). Microsomal fractions were first made 4 M guanidine HCI, 4% (w/v) Zwittergent and then treated as described elsewhere (5) before immunoprecipitation. SDS-gel electrophoresis of samples and subsequent fluorography were done as previously described (5).

**Other Procedures**—Sepharose CL-2B and CL-4B and Sephadex PD-10 chromatographic analyses were done as described elsewhere (19). Dissociative CscI-density gradient centrifugation was done as described previously (19).

### RESULTS

**Subcellular Fractionation of Chondrocytes on Sucrose Gradients**—The Golgi apparatus is the site for a number of terminal steps in the processing of proteoglycans, including the addition of O-linked oligosaccharides (18) and synthesis and sulfation (13) of glycosaminoglycan chains. After sulfation, the completed molecules are secreted from the cell with a half-time of about 5 min (19). A 2-min pulse with [35S]sulfate was chosen to identify Golgi-derived elements in the sucrose gradient (smooth microsomes), while a 10-min labeling time with [3H]serine was used to identify newly synthesized protein in rough endoplasmic reticulum fractions (rough microsomes). The distribution of macromolecular [3H] and [35S] activity in subcellular fractions prepared from chondrocytes labeled with [3H]uridine for 3 h prior to harvest. The [3H] activity present in both microsome peaks was completely labile in 0.1 M NaOH and thus present as RNA and not DNA. This indicates that rough microsomes are present in both peaks. The distribution of [3H]uridine between the two peaks was identical with that of [3H]serine (Fig. 1a) further supporting the conclusion that rough microsomes are present in both peaks. The distribution of protein (Fig. 1b) also showed a similar distribution between the two peaks, but there was an increased amount in the upper portion of the gradient, representing soluble proteins.

Glucose-6-phosphatase, while not exclusively associated with rough microsomes (33, 36), has been used in cell fractionation studies as a general marker for elements of the rough endoplasmic reticulum (37). 5'-Nucleotidase is generally considered a useful marker for the plasmalemma (36). The distribution of these two enzymes in subcellular fractions is shown in Fig. 2. While glucose-6-phosphatase activity was present in three peaks, one at each sucrose interface, 5'-nucleotidase activity was present primarily in the smooth microsome peak. The specific activity of 5'--nucleotidase in this peak was 20-fold higher than in the rough microsome peak. The specific activities of glucose-6-phosphatase in the rough microsome peak (0.084 μmol/min/mg of protein) and of 5'-nucleotidase in the smooth microsome peak (1.05 μmol/min/mg of protein) were similar to those found in the rough microsome and plasma membrane fractions of subcellular fractions from rat liver (37, 38). Neither enzyme was present in significant amounts in the soluble fractions at the top of the gradients. While these results suggest that fragments of the plasmalemma are present in the smooth microsome fractions, the general distribution of glucose-6-phosphatase could not be used to evaluate the purity of the subfractions.

**Recoveries of Markers in Subcellular Fractions**—Table I summarizes the recoveries of protein, marker enzyme activities, and radioactivities in the three indicated subcellular fractions. Recovery of [3H]uridine in macromolecules (RNA) in the postmitochondrial supernatant was greater than 95%.

![Fig. 1](http://www.jbc.org/) Distribution of labeled subcellular components on discontinuous sucrose gradients. a, postmitochondrial supernatants doubly labeled with [3H]serine (●) and [35S]sulfate (○). Sucrose molar concentrations are shown at the top of the panel, %, distribution of protein concentrations (●) and [3H] activity (○) in postmitochondrial supernatants from chondrocytes labeled with [3H]uridine. The percentages of total microsomal radioactivity (or protein) (sum of fractions 2, 3, 6, and 7) present in the rough microsomes (fractions 2 and 5) are indicated.

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\(^2\)The same distribution was observed for cultures fractionated after a 1-min serine pulse.

\(^3\)While each fraction is cross-contaminated to some extent, for convenience we will refer to the denser fraction as the rough microsome fraction and the less dense fraction as the smooth microsome fraction.

\(^4\)The resolution between the two microsome fractions was not improved by altering the densities of the sucrose layers, indicating a substantial overlap in the densities of the two microsome populations, possibly due to the contribution of proteoglycan monomer to the density of smooth microsomes. Nor was resolution improved by (a) the addition of 15 mM CsCl to the homogenization buffer (34) or (b) varying the MgCl₂ in the sucrose buffers from 5–15 mM (data not shown).
Subcellular Localization of Proteoglycan Core Protein

TABLE I

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>Lowry protein (30 min)</th>
<th>[3H]Ser (30 min)</th>
<th>[3H]Ser (240 min)</th>
<th>[35S]SO4 (2 min)</th>
<th>[3H]U (180 min)</th>
<th>G-6-Pase</th>
<th>5'-Nucleotidase</th>
<th>[3H]Man (270 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postmitochondrial supernatant*</td>
<td>83</td>
<td>64</td>
<td>85</td>
<td>49</td>
<td>88</td>
<td>93</td>
<td>94</td>
<td>68</td>
</tr>
<tr>
<td>Rough microsomes (RM)*</td>
<td>4</td>
<td>12</td>
<td>7</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Smooth microsomes (SM)*</td>
<td>10</td>
<td>19</td>
<td>23</td>
<td>64</td>
<td>12</td>
<td>28</td>
<td>41</td>
<td>28</td>
</tr>
<tr>
<td>RM/(RM + SM)</td>
<td>0.28</td>
<td>0.39</td>
<td>0.22</td>
<td>0.04</td>
<td>0.38</td>
<td>0.25</td>
<td>0.04</td>
<td>0.36</td>
</tr>
</tbody>
</table>

* Recovery expressed as per cent total activity.

Fractions 2-3, Fig. 1; recovery expressed as per cent radioactivity in gradient.

Fractions 6-7, Fig. 1; recovery expressed as per cent radioactivity in gradient.

indicating good recovery of elements of the rough endoplasmic reticulum. Recovery of macromolecular [35S] activity, 49%, was less and probably reflects loss of some Golgi elements or of secretory vesicles or of both in the mitochondrial pellet (see also Footnote 5 below).

Characterization of Proteoglycan-related Molecules Present in Microsome Fractions—Microsome fractions were isolated from chondrocytes labeled with [3H]serine for 195 min and immunoprecipitates were prepared from aliquots using an antisem (R98) containing antibodies specific to core protein and link protein (5, 33). Fractionation of [3H]labeled rough and smooth microsomes by SDS-gel electrophoresis, with subsequent fluorography, showed the presence in both fractions of a large number of labeled polypeptides throughout a wide range of molecular weights (Fig. 3, a and b). The predominant labeled species in the rough microsomes (Fig. 3a) was a high molecular weight protein which migrated slightly faster than a standard of [3H]serine-labeled aAl proteoglycan after chondroitinase ABC-treatment (Fig. 3e). This protein was specifically precipitated by the R98 antisem (Fig. 3c) and has been identified previously as the core protein precursor for the cartilage proteoglycan (5). There was no indication of proteolysis of the core protein during microsome isolation and subsequent immunoprecipitation. The major labeled molecular species in the smooth microsome fraction, on the other hand, was present at the top of the gel (Fig. 3b) and has been identified as completed proteoglycan monomer (5). It was immunoprecipitated by R98 (Fig. 3d). Core protein precursor was also present in the gel, as expected since this fraction is cross-contaminated with rough microsomes (Figs. 1 and 2).

The proportion of [3H]serine activity precipitated by R98 was 26% and 9% of the total for rough and smooth microsomes, respectively (Table II), indicating an enrichment of proteoglycan-related antigens in the rough microsome fraction.

The distribution of core protein among subcellular fractions was quantitated by SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedures." After 90 min of labeling with [3H]serine, greater than 60% of the label in total protein and greater than 70% of total labeled core protein

Fig. 2. Distribution of glucose-6-phosphatase (●) and 5'-nucleotidase (○) in discontinuous sucrose gradients. Specific activities are shown. Molar concentrations of sucrose are indicated at the top of the panel.

Fig. 3. Electrophoresis and fluorography of [3H]serine-labeled subcellular fractions on 4% polyacrylamide gels. a, rough microsome fraction; b, smooth microsome fraction; c, immunoprecipitate of rough microsomes; d, immunoprecipitate of smooth microsomes; e, standard of chondroitinase ABC-treated [3H]serine-labeled proteoglycan aggregate (aAl). Core and link proteins resulting from digestion of the standard are indicated by arrows.

TABLE II

| Immunoprecipitate of proteoglycan-related antigens from microsome fractions |
|-----------------------------|-----------------|-----------------|-----------------|
|                            | [3H]Serine (195 min) | [3H]Mannose (270 min) | [3H]Glucose (270 min) |
| Rough*                     | 26              | 3               | 6               |
| Smooth*                    | 9               | 1               | 3               |
| Rough/(rough + smooth)     | 0.66            | 0.59            | 0.25            |

* Recovery expressed as per cent total radioactivity in fraction.
were recovered in the postmitochondrial supernatant in the fractions from the gradient (Table III). Core protein precursor was most enriched in the rough microsomes, where it accounted for nearly 10% of the \(^3\)H activity and where 15% of the total intracellular core protein precursor was recovered. The large amount, 39%, of core protein present in the soluble fractions of the sucrose gradient (fractions 11–16, Fig. 1) was consistent with the results of Scheele et al. (25) who reported leakage of up to 40% of the labeled contents of rough microsomes during the fractionation procedure.

Characterization of Proteoglycan-related Molecules Present in Microsome Fractions Isolated from Cells Labeled with Different Precursors—Immunoprecipitates were obtained from rough and smooth microsome fractions isolated from chondrocytes labeled with either \([^{1}H]m\)annose or \([^{3}H]g\)lucose for 270 min. SDS-gel electrophoresis and fluorography of the \([^{1}H]m\)annose-labeled immunoprecipitates (Fig. 4) showed a pattern of macromolecules similar to those containing \([^{3}H]\)serine (Fig. 3). The presence of mannose-labeled core protein and link protein in the rough microsome fraction indicated that the addition of mannose oligosaccharides was initiated on these proteins while they were still in the rough endoplasmic reticulum. Immunoprecipitates solubilized in 4 M guanidine HCl, 0.5% Triton X-100 were chromatographed on Sepharose CL-4B and resolved as three peaks (Fig. 5, a and b). SDS-polyacrylamide gel electrophoresis of pooled fractions from each peak indicated that (a) the void volume peak corresponded to completed monomer, (b) the included peak at \(K_{\alpha} = 0.41\) contained primarily core protein-precursor, and (c) the included peak at \(K_{\alpha} = 0.76\) contained primarily link protein. Labeled core protein precursor was a major constituent of both the immunoprecipitates of rough and smooth microsome preparations, comprising about 69% and 59%, respectively, of the total \(^3\)H activity. The smooth microsome immunoprecipitate was enriched in labeled completed monomer relative to the immunoprecipitate from rough microsomes. The ratio of link protein to core protein precursor plus monomer, 16–18%, was the same for each fraction. Previous experiments have shown that between 75% and 90% of the \(^3\)H activity in proteoglycans recovered from \([^{1}H]m\)annose-labeled cells remains in mannose (6, 39), with the variation occurring when different batches of \([^{1}H]\)mannose are used.

Glucose is a precursor for both glucuronic acid and hexosamines which are incorporated into glycosaminoglycans when they are synthesized in the Golgi. Chromatography of immunoprecipitates of rough and smooth microsomes from cells labeled with \([^{3}H]\)glucose gave very different results from those labeled with \([^{1}H]m\)annose. Incorporation into molecules immunoprecipitated from the rough microsome fraction was minimal (Fig. 5c); the \(^3\)H activity associated with core protein may represent glucose residues present in the high mannose oligosaccharides (12). In the smooth microsome immunoprecipitates, however, over 75% of the \(^3\)H activity eluted as completed monomer in the void volume (Fig. 5d).

**Table III**

<table>
<thead>
<tr>
<th>Subcellular fractions*</th>
<th>% total (^3)H</th>
<th>% (^3)H in core</th>
<th>% of total core</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–3 (rough)</td>
<td>4.2</td>
<td>9.5</td>
<td>14.8</td>
</tr>
<tr>
<td>4–5</td>
<td>1.7</td>
<td>6.5</td>
<td>4.1</td>
</tr>
<tr>
<td>6–7 (smooth)</td>
<td>11.1</td>
<td>3.4</td>
<td>13.9</td>
</tr>
<tr>
<td>11–16</td>
<td>51.2</td>
<td>2.1</td>
<td>39.2</td>
</tr>
<tr>
<td>Pellet</td>
<td>38.1</td>
<td>2.0</td>
<td>28.0</td>
</tr>
</tbody>
</table>

*See Fig. 1.

*Calculated from core protein peak on SDS gels.

Although the recovery of \([^{1}H]m\)annose as proteoglycan-related antigens in the immunoprecipitates was low compared to \([^{3}H]\)serine (Table II), the distributions between rough and smooth microsome fractions were very similar (59–66%). In contrast, the distribution for \([^{3}H]\)glucose (25%) was quite different, reflecting the greater utilization of this precursor
for a number of sugar residues which are incorporated into proteoglycans in the Golgi.

Rough and smooth microsome fractions from chondrocytes labeled for 5 min with \[^{35}\text{S}\]sulfate were solubilized in 4 M guanidine HCl, 4% Zwittergent (10) and portions of each were chromatographed on Sepharose CL-2B in 4 M guanidine HCl. The elution profiles of the \[^{35}\text{S}\]-labeled proteoglycans were identical (data not shown) and were equivalent to those published previously (10). Thus, there was no indication of monomers with partially completed glycosaminoglycan chains in either microsome fraction.

Flow of Proteoglycan Core Protein and Monomer through Subcellular Fractions—The cell fractionation procedures discussed above were used in an analysis of the kinetics of the movement of the core protein precursor from the rough microsomes to the smooth microsomes and the subsequent secretion of completed proteoglycan into the cell culture medium. Rough microsomes, smooth microsomes, and culture medium were recovered from chondrocyte cultures which were pulse-labeled with \[^{3}\text{H}\]serine for 30 min and then chased for various times from 0-300 min in medium without labeled precursor and containing cycloheximide to inhibit further precursor incorporation. Over the 300-min chase time there was a net loss of \[^{3}\text{H}\]activity in the rough microsomes relative to total \[^{3}\text{H}\] activity in the gel. Cross-hatched peaks indicate core protein. The percentages shown are core protein relative to total \[^{3}\text{H}\] activity in the gel.

Core protein was quantitated by SDS-polyacrylamide gel electrophoresis of rough and smooth microsome fractions from each time point (Fig. 6). The proportion of total activity present as core protein (cross-hatched peaks) in the rough microsome fraction was maximal (7.7%) at the 15-min chase time and then decreased with increasing time. The ratio of core protein in the rough microsomes to total microsomal core protein was 0.55, 0.59, 0.43, 0.48, 0.60, and 0.56 for times between 0 min and 300 min (mean = 0.54 ± 0.07). Therefore, while core protein was lost from each microsomal fraction with time, the distribution of core between rough and smooth microsomes remained essentially constant. These data are consistent with the data above (Figs. 1 and 2) indicating that the smooth microsome fraction also contains a significant proportion, 60%, of the rough microsomes. Thus, the core protein precursor in each is actually derived from the same pool of molecules in the rough endoplasmic reticulum.

The radioactivity in completed monomer for each chase time was estimated by the total recoveries in the D1 fractions (derived from data in Table IV). These values were then fit to first order equations for loss of material from a single pool of molecules (derived from data in Table IV). These values were then fit to a single pool of molecules, and the kinetic disappearance from this compartment will be representative of the entire population. These values (derived from Table IV) for core protein precursor recovered in the combined microsome fractions were fit to first order equations for loss of material from a precursor pool and yielded a \( t_{1/2} = 60 ± 8 \) min. These two values are nearly the same and consistent with a precursor-product relationship.

In order to estimate kinetics for a three-compartment system, i.e. rough microsome to smooth microsome to culture medium, a number of corrections were applied to the data in Table IV due to (a) the incomplete resolution of rough and smooth microsomes in the sucrose gradient, (b) cross-contamination of fractions with matrix components, and (c) incom-

**TABLE IV**

<table>
<thead>
<tr>
<th>Chase time</th>
<th>Total dpm [^{3}\text{H}] ( \times 10^4 ) (core + monomer)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.29 Core*</td>
<td>42.8</td>
</tr>
<tr>
<td></td>
<td>Monomer(^b)</td>
<td>4.8</td>
</tr>
<tr>
<td>15</td>
<td>3.10 Core</td>
<td>44.1</td>
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<tr>
<td></td>
<td>Monomer(^b)</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>300</td>
<td>15.94 Core</td>
<td>2.9</td>
</tr>
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</table>

\(^a\) Disintegrations/min in core calculated by (% of disintegrations/min in core peak on SDS-polyacrylamide gel electrophoresis) \( \times \) (total disintegrations/min in microsome fraction).

\(^b\) Disintegrations/min in monomer calculated by (% of disintegrations/min in D1) \( \times \) (total disintegrations/min in monomer fraction) for microsomes and by (disintegrations/min in D1) for medium.

Rough and smooth microsome fractions from chondrocytes were solubilized in 4 M guanidine HCl, 4% Zwittergent (10) and portions of each were chromatographed on Sepharose CL-2B in 4 M guanidine HCl. The elution profiles of the \[^{35}\text{S}\]-labeled proteoglycans were identical (data not shown) and were equivalent to those published previously (10). Thus, there was no indication of monomers with partially completed glycosaminoglycan chains in either microsome fraction.

**Flow of Proteoglycan Core Protein and Monomer through Subcellular Fractions**—The cell fractionation procedures discussed above were used in an analysis of the kinetics of the movement of the core protein precursor from the rough microsomes to the smooth microsomes and the subsequent secretion of completed proteoglycan into the cell culture medium. Rough microsomes, smooth microsomes, and culture medium were recovered from chondrocyte cultures which were pulse-labeled with \[^{3}\text{H}\]serine for 30 min and then chased for various times from 0-300 min in medium without labeled precursor and containing cycloheximide to inhibit further precursor incorporation. Over the 300-min chase time there was a net loss of \[^{3}\text{H}\] activity in the rough microsomes relative to the smooth microsomes (from 28% to 18% of the total microsomal activity) and also a net loss in total \[^{3}\text{H}\] activity in each microsome fraction (about 40% and 20% for rough and smooth fractions, respectively).

Core protein was quantitated by SDS-polyacrylamide gel electrophoresis of rough and smooth microsome fractions from each time point (Fig. 6). The proportion of total activity present as core protein (cross-hatched peaks) in the rough microsome fraction was maximal (7.7%) at the 15-min chase time and then decreased with increasing time. The ratio of core protein in the rough microsomes to total microsomal core protein was 0.55, 0.59, 0.43, 0.48, 0.60, and 0.56 for times between 0 min and 300 min (mean = 0.54 ± 0.07). Therefore, while core protein was lost from each microsomal fraction with time, the distribution of core between rough and smooth microsomes remained essentially constant. These data are consistent with the data above (Figs. 1 and 2) indicating that the smooth microsome fraction also contains a significant proportion, 60%, of the rough microsomes. Thus, the core protein precursor in each is actually derived from the same pool of molecules in the rough endoplasmic reticulum.

The radioactivity in completed monomer for each chase time was estimated by the total recoveries in the D1 fractions (derived from data in Table IV). These values were then fit to first order equations for the appearance of label in monomer and gave a \( t_{1/2} = 58 ± 13 \) min. While recovery of counts in completed monomer was nearly quantitative, recovery of counts in core protein precursor are low (see Table III and below). However, if the proportion of labeled core protein precursor recovered in the combined microsome fractions is constant with time, then the kinetics of disappearance from this compartment will be representative of the entire population. These values (derived from Table IV) for core protein precursor recovered in the combined microsome fractions were fit to first order equations for loss of material from a precursor pool and yielded a \( t_{1/2} = 60 ± 8 \) min. These two values are nearly the same and consistent with a precursor-product relationship.

In order to estimate kinetics for a three-compartment system, i.e. rough microsome to smooth microsome to culture medium, a number of corrections were applied to the data in Table IV due to (a) the incomplete resolution of rough and smooth microsomes in the sucrose gradient, (b) cross-contamination of fractions with matrix components, and (c) incom-
were not used to correct for the incomplete recovery of core protein precursor, the $t_{1/2}$ calculated was 57 ± 1 min. Thus, 57 min represents a lower limit on the core protein half-time. The $t_{1/2}$ of 10 ± 4 min for the smooth microsome compartment agrees well with the kinetics of $[^{35}S]$sulfate incorporation which gave a half-time of 8 min for secretion of $^{35}S$-labeled proteoglycans in the presence of cycloheximide (10).

During the 300-min chase period, the total $^3$H activity recovered in the three fractions increased 2-fold (Fig. 7a). This increase was reduced from a 5-fold increase (Table IV) which reflected the differential recoveries of core protein and monomer and which was corrected using the data in Table III. Although a low level of protein synthesis occurs in the presence of cycloheximide (approximately 5%, see Ref. 10), over the 300-min chase time, it is difficult to determine to what extent this synthesis contributed to the remaining increase in total activity.

**DISCUSSION**

Subcellular fractionation has been used frequently to study the movement of secretory proteins from their site of synthesis in the rough endoplasmic reticulum until secretion. The techniques for cell fractionation were developed largely for the rat liver and guinea pig pancreas and much of the kinetic data for the intracellular transport and secretion of exocrine proteins is a result of pulse-chase experiments using these tissues (40). More recent studies have fractionated cells as a means of determining where in the cell post-translational modifications of proteins, such as glycosylation, occur (25, 41–43). These techniques have been applied to cartilage tissue to study the localization of glycosaminoglycan synthetic enzymes (21), cartilage proteoglycan sulfation (13), and procollagen processing (24). In this paper, we adapted the subcellular fractionation method of Oohira et al. (24) to monolayer cultures of chondrocytes from the Swarm rat chondrosarcoma. The distributions of newly synthesized protein and sulfated proteoglycans between rough and smooth microsome fractions obtained from the rat chondrocytes (Fig. 1) are very similar to those reported for chick epiphyseal cartilage (14, 24).

The cell fractionation procedure used yielded a rough microsome fraction with minimal cross-contamination from Golgi, plasma membrane, or matrix components. For example: (a) after a 2-min pulse with $[^{35}S]$sulfate, the rough microsomes contained only 4% of the total microsomal $^{35}S$-labeled macromolecules some of which were probably due to absorption of completed $^{35}S$-macromolecules (23), and (b) the rough microsome fraction contained only 4% of the total microsomal 5'-nucleotidase, a plasmalemma marker. On the other hand, the smooth microsome fraction contained a constant proportion of the rough microsomes. For example, 62% of the total microsomal $^3$H activity was recovered in this fraction after a 3-h pulse with $[^3H]$uridine to label ribosomes.

In a pulse-chase experiment with $[^3H]$serine, the rough microsome fraction contained a constant proportion (53 ±
7%) of the labeled core protein precursor in the total microsomal fraction at all times. This suggests that the labeled core protein precursor in both the rough and smooth membrane fractions originated from the same pool, namely the rough endoplasmic reticulum, especially since, as was shown above, there is a considerable contamination of rough microsomes in the smooth membrane fraction.

Analysis of the loss of labeled core protein precursor from the microsomal compartment by computer fitting the data to a first order equation gave a $t_{1/2}$ of 60 ± 8 min, close to the $t_{1/2}$ calculated for the appearance of label in completed proteoglycan, as determined by independent methods. This is consistent with the precursor-product relationship of these two macromolecules. When corrections were made to estimate losses and a three-compartment model employed as described under "Results," estimates for the $t_{1/2}$ of the core protein precursor increased to 98 ± 7 min and a value for the $t_{1/2}$ = 10 ± 4 min of completed proteoglycan in the intracellular compartment (Golgi plus secretory vacuoles) was obtained. The values for the $t_{1/2}$ of the core protein precursor (60–100 min) and for the intracellular $t_{1/2}$ of the completed molecules agree well with previous estimates derived from independent methods. For example, Kimura et al. (5) demonstrated the presence of a large pool of core protein in the Swarm rat chondrocytes prior to the addition of chondroitin sulfate chains, by pulsing cells with $[3H]$serine and immunoprecipitating core protein during a 340-min chase in the presence of the cells (10–15 min).

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