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Preparation of Subcellular Fractions from Granulation Tissue by Density Gradient Centrifugation

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The 7000 *g* pellet of homogenized mature sponge-induced granulation tissue was fractionated by centrifugation in a stepwise sucrose gradient in order to study the synthesis and secretion of collagen and other components of this tissue on the subcellular level. As indicated by chemical and enzymatic assays, by electron microscopy and by incorporation experiments, the collagen-synthesizing rough endoplasmic reticulum fraction was isolated free from the secretory vesicles (smooth endoplasmic membranes and Golgi elements) and fibrous extracellular matrix. Collagen differed from other proteins in the distribution among the subcellular fractions. In pulse-chase experiment the translocation of ^{14}C-labelled collagen was demonstrated from the rough endoplasmic reticulum through the secretory vesicles to extracellular fibrillar collagen. This fractionation method will be used to study the modulation of collagen synthesis and secretion in the reparative tissue.

Differential centrifugation methods have been developed for the subcellular fractionation of matrix-free cartilage and tendon cells to obtain the rough and smooth endoplasmic reticulum.[1,2] A density gradient centrifugation was applied to get from lung tissue the rough endoplasmic reticulum containing the greatest activities of collagen prolyl and lysyl hydroxylases.[3,4] Likewise, in the microsomal fractions from chick-embryo liver the highest activity of collagen prolyl hydroxylase was found specifically located in the cisternae of rough endoplasmic reticulum.[5,6]

This study is an extension of our earlier work on the preparation of plasma membranes from the fibroblasts of the experimental granulation tissue,[7] which produces, among other proteins, collagen types I and III.[8] The experimental granulation tissue can be used to investigate the activation and differentiation of connective tissue, while the matrix-free cartilage and tendon cells are not suitable for such studies. The main purpose of this work was to develop methods for the further studies on the regulation of the synthesis and secretion of collagen, other proteins and carbohydrates of the integrated granulation tissue at the subcellular level.

MATERIALS AND METHODS

Production of granulation tissue and incubation of the slices with labelled proline. Female albino rats of the Wistar strain (2 – 3 months old, weight 130 – 200 g) were kept on a standard laboratory diet (from Messrs. Hankkija, Helsinki, Finland) supplemented with vegetables. Growth of the granulation tissue was induced by well-washed pieces of viscose-cellulose sponge (dry weight 60 – 70 mg, dry size 4 × 14 × 14 mm, from Kongsfoss Fabrikker A/S, Bygdøy allé 5, Oslo, Norway). The sponge pieces were sterilized by boiling in 0.9 % NaCl and implanted symmetrically under the dorsal skin of each rat.[9] The granulomas were removed in the mature, *i.e.*, collagen-synthesizing phase after an implantation period of 21 days.[10]

The granuloma slices were prepared and incubated *in vitro*, as described in detail by Aalto *et al.*,[11] in Krebs-Ringer-Hepes medium buffered at pH 7.4, and supplemented with 22.4 mM glucose and 2.87 mM proline. When phosphate determinations were to be made no phosphate was added to the buffer. Each sample consisted of 10 g (wet weight) of granuloma slices in 60 ml of the medium. After a 15 min preincubation, 3.3 μCi of L-[^{3}H]proline (TRA. 82, The Radiochemical Centre, Amersham, Bucks., U.K.) per 1 ml medium was added and the incubation continued for a further 20 min, 1 h or 3 h. To stop the incorpora-

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tion of proline, 5.4 mg of cycloheximide (Sigma No. C-6255) and 8.0 mg of 2,2'-bipyridine (Merck No. 3098) were added. All samples were kept at 37 °C for 3 h. The slices were separated from the medium by filtering the incubation mixture through a double thickness of nylon cloth.

In some experimental series the granuloma slices were pulse-labelled for 20 min, after a 15 min preincubation, with L-[^{3}H]proline (6.6 μCi ml^{-1} medium). The ^{3}H-labelled polypeptides were chased with unlabelled proline (0.83 mg/ml medium) and the incubation continued for up to 1 h, 3 h and 5 h. Another series with pulse-labelling was performed with 4 μCi of L-[^{14}C]proline (CFB.71, The Radiochemical Centre, Amersham, Bucks., U.K.) per ml of the medium. After a pulse of 5 min the medium was decanted off and the slices put into a fresh medium containing 100 μg/ml of cold proline. The incorporated label was chased for 5 min, 15 min and 35 min. To stop the chase period the samples were immersed in crushed ice. Further protein synthesis was inhibited by the addition of cycloheximide, final concn. 100 μg/ml.

Subcellular fractionation of the incubated slices. The granuloma slices were homogenized and fractionated (MSE 65, 6 × 18 ml swing-out rotor) as described previously [7] except that the discontinuous gradient contained 20, 28, 38, 45, 55, 65 and 70 % (w/v) sucrose. The media from the incubations of the granulation-tissue slices and the 7000 *g* supernatant of the granulation tissue homogenate were centrifuged at 100 000 *g* for 45 min (MSE 50, 8 × 25 ml).

The differential centrifugation of the homogenized granuloma slices (Table 3) was carried out as described by Harwood *et al.*[1]

Enzyme assays. The following enzymes were determined for the characterization of the subcellular fractions: 5'-Nucleotidase,[12] S.D. ± 10.8 %; acid phosphatase,[13] S.D. ± 9.6 %; β-glycerophosphate was used as the substrate and alkaline phosphatase was inhibited with 4 mM EDTA;[14] Na^+,K^+-activated Mg^{2+}-dependent adenosine triphosphatase,[15] S.D. ± 6.5 %; glucose-6-phosphatase,[16] S.D. ± 7.0 %; the acid and alkaline phosphatases were inhibited with 2 mM KF and 4 mM EDTA, respectively;[14] leucine-β-naphthylamidase,[17] S.D. ± 8.5 %; succinate dehydrogenase,[18] S.D. ± 15.0 %. Inorganic phosphate, liberated in the enzyme assays, was determined as proposed by Martin and Doty,[19] S.D. ± 4.6 %.

The specific activities of the enzymes were expressed as the amount of reaction product liberated at 37 °C in μmol mg^{-1} protein, determined according to Lowry *et al.*,[20] S.D. = 2.0 %.

Determination of nucleic acids and cholesterol. The tissue fractions were diluted with one volume of distilled water and centrifuged at 100 000 *g* for 45 min. The sedimented pellets were washed three times with 80 % ethanol to remove sucrose, then dried and nucleic acids fractionated.[21] RNA was estimated by determining phosphate as described by Bartlett [22] after the digestion according to Svanborg and Svennerholm.[23] DNA was assessed by the method of Burton.[24]

For the determination of cholesterol, a significant constituent in membranes, tissue fractions were sedimented and washed twice with water by centrifuging at 100 000 *g* for 40 min. The pellet was freeze-dried and extracted three times for 24 h with chloroform-methanol (2:1, v/v) at room temperature.[25] Cholesterol was determined as described by Badzio and Doczon.[26]

Electron microscopy. The sedimented samples were fixed overnight in 3 % glutaraldehyde in 0.2 M sodium dimethylarsinate buffer, pH 7.25, post-fixed for 30 min in 1 % OsO_4 in the same buffer and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead hydroxide and studied in a Siemens Elmiskop 1A electron microscope (Laboratory of Electron Microscopy, University of Turku).

Assay of radioactivity. The sedimented pellets, obtained as described above, were washed five times with 80 % ethanol and hydrolyzed in 6 N HCl for 3 h at 130 °C. The hydrolysates were evaporated to dryness over a boiling water bath and redissolved in 8 ml of water. An aliquot was taken for nitrogen determination.[27,28] For the determination of the total radioactivity, a portion was pipetted into 10 ml of scintillant consisting of 3.75 ml ethylene glycol monomethyl ether (Merck) and 6.25 ml scintillation liquid containing 15 g of 2,5-diphenyloxazole (PPO, Packard, No 6002026) and 50 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (POPOP, Packard No 6002030) in 1000 ml of distilled toluene. To determine the content of hydroxylated collagen in the tissue fractions radioactive hydroxyproline was assayed by the method of Juva and Prockop.[29] The amount of total radioactive collagen, without regard to the degree of hydroxylation, was estimated by using bacterial collagenase.[30] The radioactivities were measured with a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3320. The counting efficiency was 25 % for ^{3}H and 80 % for ^{14}C.

RESULTS

Composition and identification of the subcellular fractions. The 7000 *g* pellet of the homogenized granulation-tissue slices contains the main part of the nucleic acids and hydroxylated collagen but only 12 % of the total protein of the slices (Table 1). There are marked differences in the distribution of the total protein and collagen, especially at the interphases of 45/55 % and 55/65 % sucrose and in the bottom (> 70 % sucrose) of the tube

Table 1. Composition of the subcellular fractions from the incubated granuloma slices. Slices of 3-wk granulation tissue were incubated in Krebs-Ringer-Hepes buffer, pH 7.4, at 37 °C for 3 h, homogenized in iso-osmotic sucrose and centrifuged at 7000 *g* for 30 min. The supernatant was centrifuged at 100 000 *g* for 45 min. The 7000 *g* pellet was rehomogenized in iso-osmotic sucrose and applied into a discontinuous sucrose gradient (20, 28, 38, 45, 55, 65 and 70 % w/v) and centrifuged at 100 000 *g* for 4 h in a swing-out rotor. The yields are not corrected. Their sums are less than 100 % because of losses during the fractionation.

Subcellular fraction of slices	Yield/%				Identification
	DNA	RNA	Protein	Collagen (hydroxylated)[a]	(*cf.* Table 2)
Homogenized slices	100	100	100	100	–
7000 *g* supernatant	21	36	55	35	–
100 000 *g* pellet	13	11	4	10	"Microsomes"
100 000 *g* supernatant	8	25	51	25	Cytosol
7000 *g* pellet	72	58	12	51	–
7000 *g* pellet in sucrose gradient					
Top layer (soluble)	1	3	3	2 (32)	Cytosol
Top layer/20 %	6	3		(24)	
20/28 %	5	4	1	1 (35)	Plasma membranes, Golgi- and other vesicles
28/38 %	4	6	2	3 (30)	
38/45 %	3	6	0.4	5 (25)	Mitochondria, smooth endoplasmic reticulum
45/55 %	7	18	1.5	7 (29)	Rough endoplasmic reticulum
55/65 %	13	10	0.6	14 (14)	
65/70 %	13	6	0.3	6 (25)	
>70 %	20	4	0.5	13 (21)	Nuclei, collagenous fibrils, insoluble proteins, debris

[a] Hydroxylation/% in the parentheses.

Table 2. Marker enzymes in the subcellular fractions from the incubated slices. Slices of 3-wk granulation tissue were incubated, homogenized and fractionated as explained in the legend of Table 1 and in the Material and Methods section. The relative specific activity refers to the original homogenate. n.d. = not determined.

Subcellular fraction of slices	5′-Nucleotidase		ATPase		Leucine-β naphthyl-amidase		Succinate dehydro-genase		Glucose-6-phosphatase		Acid phosphatase	
	Yield/ %	Rel.sp. act.	Yield/ %	Rel.sp. act.	Yield/ %	Rel.sp. act.	Yield/ %	Rel.sp. act.	Yield/ %	Rel.sp. act.	Yield/ %	Rel.sp. act.
Homogenized slices	100	1.0	100	1.0	100	1.0	100	1.0	100	1.0	100	1.0
7000 *g* supernatant												
100 000 *g* pellet	15	3.9	11	3.0	4	1.1	n.d.	—	5	1.4	6	1.6
100 000 *g* supernatant	60	0.6	19	0.4	15	0.3	n.d.	—	93	1.9	44	0.9
7000 *g* pellet	27	2.2	39	3.2	34	2.8	n.d.	—	20	1.6	24	2.1
7000 *g* pellet in sucrose gradient												
Top layer (soluble), <20 %	3	1.3	6	2.4	6	2.4	<2	0.0	3	1.1	4	1.6
20/28 %	3	2.9	8	9.0	7	8.3	<2	0.0	3	1.8	4	3.5
28/38 %	5	3.1	16	10.1	11	7.3	<2	0.0	3	1.6	6	3.9
38/45 %	2	3.9	2	5.3	1	2.5	15	33.0	2	5.3	3	7.0
45/55 %	1	0.9	2	1.4	1	0.7	13	9.6	2	1.7	2	1.7
55/65 %	0	0.7	1	1.8	1	1.1	<2	4.2	1	2.1	1	1.8
65/70 %	0	0.8	0	1.9	0	1.1	<2	0.0	0	1.8	0	1.0
>70 %	0	0.9	1	1.1	0	0.8	<2	0.0	0	1.1	0	0.7
Ratio medium/slices	2.0	2.5	0.4	0.4	0.5	0.4	—	—	5.0	6.7	1.1	1.3

(see also Fig. 2). The bulk of RNA (28 %) and of the hydroxylated collagen (21 %) concentrates at the combined interphases of 45/55 % and 55/65 % sucrose suggesting that it contains the rough endoplasmic reticulum. Electron microscopic examination (not presented) confirmed this identification. The main DNA, probably in nuclei and their fragments, sediments through the 70 % sucrose. 13 % of the hydroxylated collagen sediments through 70 % sucrose. Soluble collagen does not penetrate into 20 % sucrose, but fibrillar rat tail tendon collagen sediments partly to the interphase of 65/70 % sucrose and mainly in the bottom of the tube.[31] In electron microscopy cross-striated fibrillar structures were observed but none with distinct collagen bands. Thus, collagen in this fraction is presumably in the solid fibrillar form. The incorporation experiments (Fig. 2 and 4) confirmed the identification of rough endoplasmic reticulum.

The collagen content of the various subcellular fractions was estimated also employing collagenase digestion,[30] so that non-hydroxylated collagen is included. When the results were compared with those based on the content of hydroxyproline, it was found, in two independent series, that the 55/65 %-sucrose fraction contained proportionally more non-hydroxylated collagen than the other fractions (Table 1).

This is in agreement with the conclusion that collagen chains are synthesized there. When a "crude" endoplasmic reticulum preparation, sedimenting to the interphase of 45/65 % sucrose, was recentrifuged in a linear sucrose gradient, a single maximum for collagen synthesis was located at about 50 % sucrose, while the maximum synthesis of total protein was observed at 42 % sucrose.

Table 2 shows the distribution and specific activities of the marker enzymes in the same fractions. The determinations were made on fractions from homogenized incubated slices so as to be useful for the evaluation of the results of the incorporation experiments. Na^+,K^+-activated, Mg^{2+}-dependent adenosine triphosphatase was, under these conditions, a better marker for plasma membranes than the more soluble 5′-nucleotidase. The location of mitochondria was clear-cut as indicated by the activity of succinate dehydrogenase. The

Table 3. Distribution of the subcellular fractions, obtained from experimental granulation tissue by differential centrifugation, in the sucrose gradient. The fractions (nuclear, mitochondrial, rough endoplasmic reticulum and smooth endoplasmic reticulum) were obtained by differential centrifugation as described by Harwood *et al.*[1] Each fraction was then centrifuged at 100 000 *g* for 4 h in a swing-out rotor in a discontinuous sucrose gradient (20, 28, 38, 45, 55, 65 and 70 % w/v). Yield in %.

Fraction	Nuclear		Mitochondrial		Rough endoplasmic reticulum			Smooth endoplasmic reticulum		
Conc. of sucrose/%	Nitrogen	DNA	Nitrogen	Cholesterol	Nitrogen	RNA	Cholesterol	Nitrogen	RNA	Cholesterol
Top layer (soluble)	2	0	4	5	0	1	24	5	4	30
<20	7	3	3	2	0	2	0	2	4	23
20/28	4	11	4	4	33	15	0	14	12	36
28/38	2	6	6	20	30	18	24	46	26	0
38/45	25	12	53	57	10	41	18	20	42	11
45/55	17	12	27	7	26	11	35	0	6	0
55/65	24	19	0	2	0	2	0	11	2	0
65/70	13	13	0	2	0	1	0	0	2	0
>70	4	25	1	1	0	0	0	0	0	0

activity of acid phosphatase indicates that lysosomes sedimented mainly to the interphases of 28/38 % and 38/45 % sucrose, *i.e.*, in part with the plasma membrane fractions (28/38 % sucrose interphase). The smooth endoplasmic membranes were localized mainly at the interphase of 38/45 % sucrose as judged from the specific activity of glucose-6-phosphatase.

Comparison of differential and isopycnic centrifugation. The nuclear, mitochondrial, rough and smooth endoplasmic reticulum fractions obtained from the granulation-tissue homogenate by the conventional differential centrifugation[1] were each refractionated as described above in the discontinuous sucrose gradient (Table 3). All the original subcellular fractions from the differential centrifugation were divided among several sucrose interphases and thus proved to be heterogenous by density (see Discussion).

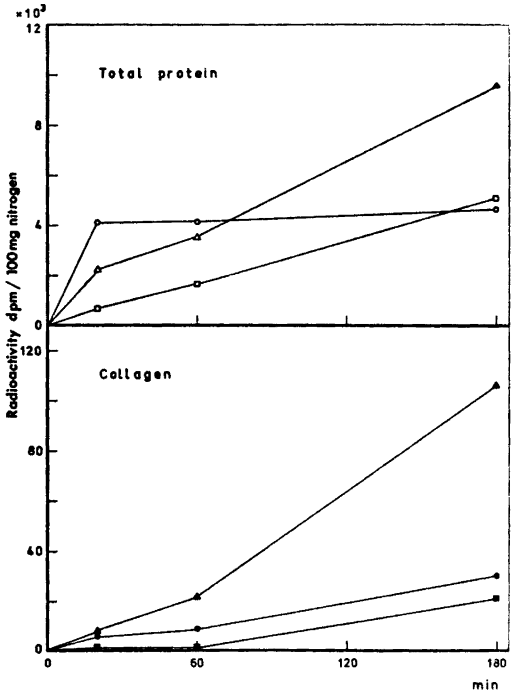

Fig. 1. Time course of the incorporation of proline to collagen and other proteins in the different compartments at the incubation of granulation-tissue slices. Slices of 3-wk granulation tissue were incubated in the presence of 3.3 μCi of L-[^{3}H]proline. The values are averages of three independent experiments. ○ ● Medium; △ ▲ homogenized slices; and □ ■ 100 000 *g* supernatant of the homogenized slices. The open symbols indicate total protein and the closed symbols collagen.

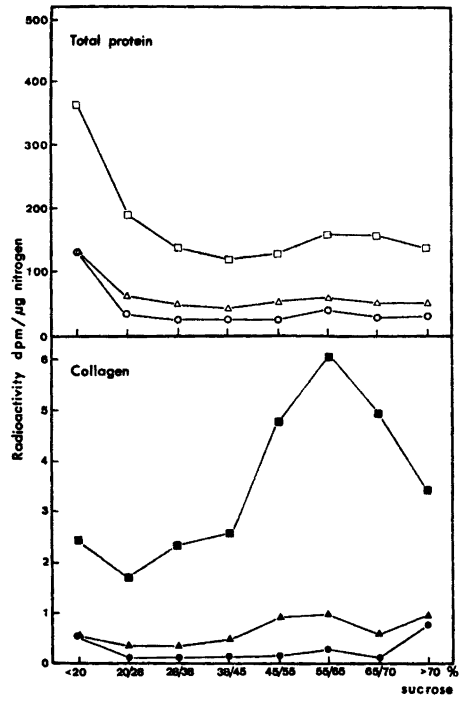

Fig. 2. Incorporation of labelled proline to the sucrose gradient fractions of the 7000 *g* pellet of homogenized granulation-tissue slices. Other data on the same material are given in Fig. 1. The values are averages of three independent experiments. Incorporation to the subcellular fractions ○ ● at 20 min; △ ▲ at 60 min; and □ ■ at 180 min. The open symbols indicate total protein and the closed symbols collagen.

Incorporation of proline to various subcellular fractions. The incorporation of [^{3}H]proline from the medium into the incubated slices and cytosol fraction was almost linear with time up to 3 h (Fig. 1). The radioactivities in the 100 000 *g* sediments of both the medium and the 7000 *g* supernatant of the homogenized slices were not significant and therefore are not presented. The labelled total protein in the medium reached its final level during the first 20 min. The shape of the curves for labelled collagen was somewhat different. The main part of collagen is found in the 7000 *g* pellet of the homogenized slices. The steepest part of the

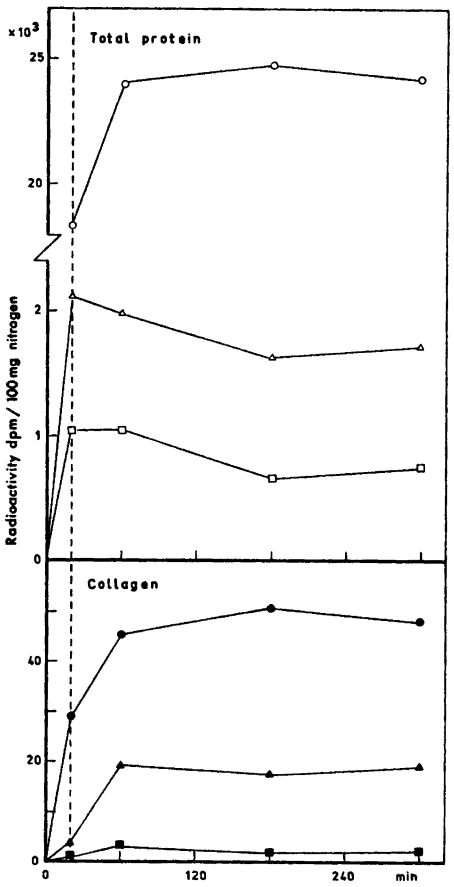

Fig. 3. Pulse-experiment on the incorporation of L-[^{3}H] proline to collagen and other proteins in the different compartments at the incubation of granulation-tissue slices. Slices of 3-wk granulation tissue were pulse-labelled for 20 min with 6.6 μCi of L-[^{3}H]proline. ○ ● Medium; △ ▲ homogenized slices; and □ ■ 100 000 *g*-supernatant of the homogenized slices. The open symbols indicate total protein and the closed symbols collagen.

curve intersects time-axis at about 30 min, presumably because there is a time lag due to hydroxylation. The ratio for the radioactivity in the 7000 *g* supernatant/pellet increases during the 3 h incubation from 1.4 to 2.0 for total protein but remains a steady 0.4 for collagen (not presented in detail).

The corresponding time dependence for the accumulation of radioactivity in the subcellular particles is shown in Fig. 2. The difference in the distributions of radioactivities of total protein and collagen is marked. The abundance of total protein was located at the soluble fraction (< 20 % sucrose). Collagen is preferentially found at the interphases of 45/55, 55/65 and 65/70 % sucrose, which were identified to contain the rough endoplasmic reticulum.

When the granulation-tissue slices were pulse-labelled for 20 min with [^{3}H]proline and transferred to fresh medium that contains non-labelled proline (Fig. 3) there was no further marked increase in the incorporated radioactivity in total protein. In the collagen moiety there is an apparent increase up to 60 min, probably because the hydroxylation of the collagen peptide continues.

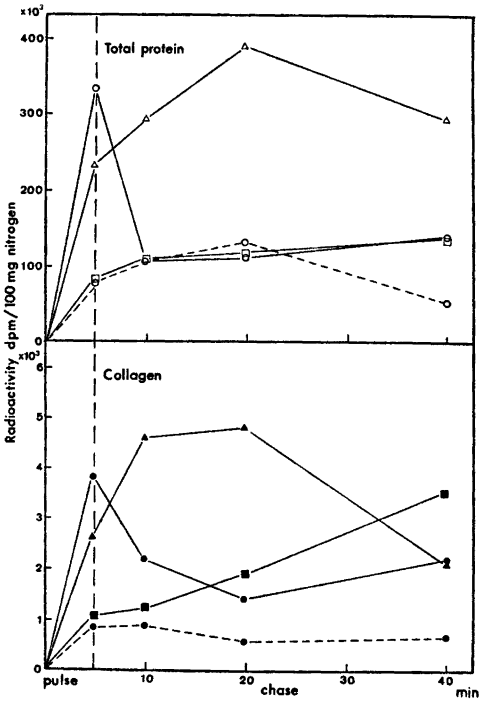

Fig. 4. Translocation of ^{14}C-labelled collagen and other proteins through the subcellular compartments at the incubation of the granulation-tissue slices. ○ ● Combined interphases of 45/55, 55/65 and 65/70 %-sucrose (the rough endoplasmic reticulum); △ ▲ combined interphases of 20/28, 28/38 and 38/45 %-sucrose (the secretory vesicles); □ ■ > 70 %-sucrose (insoluble materials, collagen fibrils); ---○--- ---●---, < 20 %-sucrose + 100 000 *g* supernatant (cytosol fraction). The open symbols indicate total protein and the closed ones collagen.

Because there was no more secretion of collagen after 60 min, the pulse-chase experiment was repeated with [^{14}C]proline and with shorter time intervals (Fig. 4). The slices were incubated for 5 min with [^{14}C]proline followed by a chase with unlabelled proline for up to 35 min. A passage of [^{14}C]proline-labelled polypeptides through the subcellular compartments of the granulation-tissue cells could then be shown. The maximum labelling of both total protein and collagen during the 5 min pulse occurred in the rough endoplasmic reticulum fraction. The transfer of the label to the secretory vesicles reached a maximum after a 5 – 15 min chase. Only a very small proportion of the [^{14}C]hydroxyproline was recovered in the cytosol fraction. This result is in agreement with the studies of Harwood *et al.*[32] with detached cartilage and tendon cells and confirms that the cytoplasm is not a compartment in the procollagen transport system. During the 35 min chase radioactive collagen accumulated at the bottom of the gradient (> 70 % sucrose), presumably in the fibrils. On the other hand some radioactive non-collagen protein accumulated also in the cytosol fraction.

For functional purposes (see Fig. 4) the sucrose fractions were combined as follows: cytosol, 100 000 *g* supernatant + 20 % sucrose fraction; secretory vesicles, 20/28, 28/38 and 38/45 % sucrose fractions; rough endoplasmic reticulum, 45/55, 55/65 and 65/70 % sucrose fractions; fibrillar collagen, > 70 % sucrose fraction.

DISCUSSION

Previously no method has been developed to study the collagen synthesis and secretion of granulation-tissue at subcellular level. As shown in Table 3 the differential centrifugation used in most studies thus far is not very suitable for the granulation-tissue cells which contain lighter endoplasmic reticulum, presumably with less ribosomes, than, for example, the tendon cells.[1,2]

In the present experiments matrix-containing material has been used, so that the intra- and extracellular soluble materials cannot be separated. However, the collagen fibrils can be harvested in the bottom of the sucrose gradient (see Results section). The synthesis of collagen does not dominate the protein synthesis as in suspended tendon, cartilage or bone fibroblasts. The cell population is natural, albeit heterogenous. When collagen is the focus of study, the heterogeneity of cells is not a drawback. On the contrary, the use of solid, natural granulation tissue may have advantages over a suspension of detached cells where extracellular processes are no longer pertinent. When the homogeneity of cells and the absence of matrix are imperative, detached or cultured granuloma fibroblasts must be used.[33]

The identification of rough endoplasmic reticulum was further supported by the highest activities of collagen prolyl and lysyl hydroxylases in the 45/55 % sucrose fraction and by the almost equally high activities in the adjacent fractions. The maximal specific activity of collagen glucosyltransferase also was observed in the 45/55 % sucrose fraction. The activities of these hydroxylases and transferases were determined in Prof. K. Kivirikko's laboratory (Department of Medical Biochemistry, University of Oulu, Oulu, Finland).

Prof. U. Lindahl (Veterinary College, Institute for Medical Chemistry, Uppsala, Sweden) assessed the incorporation of UDP-glucuronic acid in the presence of UDP-hexosamines by the 28/38 and 45/55 % sucrose fractions. In the latter fraction, identified above as a part of the rough endoplasmic reticulum, the activity was negligible, but some activity was observed in the 28/38 % sucrose fraction suggesting the presence of Golgi apparatus in addition to plasma membranes.

When the granuloma slices are labelled continuously with radioactive proline, the hydroxylated collagen seems to accumulate in the rough endoplasmic reticulum fraction and is translocated only to a small extent (Fig. 2). The metabolic capacity for intracellular translocation, which is dependent on the supply of ATP,[34] presumably deteriorates during an incubation prolonged over 60 min, when the synthesis of collagen still continues. Microtubular inhibitors as well as a shortage of ATP are known to disturb the secretion of collagen.[32,34] In the present experiments the cells are located in slices of mature granulation tissue and it is conceivable that there could be

a feedback regulation against the transport of more collagen to the extracellular space.

Recent morphological and radioautographic studies on odontoblasts,[35] specific immunocytochemical studies with chick-tendon cells [36] and corneal fibroblasts [37] have produced evidence that procollagen is secreted through the classical route of extracellular proteins. Harwood *et al.*[32] used a subcellular fractionation approach which permits a kinetic analysis of the transfer of procollagen through the subcellular compartments. In the present work the pulse-chase studies on the subcellular fractions from granulation-tissue (Fig. 4) demonstrate a transfer of ^{14}C-labelled hydroxylated collagen from the rough endoplasmic reticulum first to the secretory vesicles (smooth microsomal membranes and Golgi-elements) and then to extra-cellular fibrillar collagen. The translocation of collagen seems to happen more rapidly in granuloma cells than in matrix-free embryonic-chick tendon, cartilage [32,38] or arterial cells.[39] It also differs from the secretion of non-collagenous protein.

These experiments do not open new possibilities for the study of collagen synthesis as such, except through the preparation of the subcellular fractions by the density gradient centrifugation. The main emphasis is in introducing the versatile granulation tissue as the biological system which differentiates and which is the subject of various regulations and drug treatments. It is more complex than tendon or cartilage but required as a whole for the production of connective tissue in wound healing.

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REFERENCES

1. Harwood, R., Grant, M. E. and Jackson, D. S. *Biochem. J. 144* (1974) 123.
2. Harwood, R., Grant, M. E. and Jackson, D. S. *Biochem. J. 152* (1975) 291.
3. Cutroneo, K. R., Guzman, N. A. and Sharawy, M. M. *J. Biol. Chem.* 249 (1974) 5989.
4. Guzman, N. A., Rojas, F. J. and Cutroneo, K. R. *Arch. Biochem. Biophys. 172* (1976) 449.
5. Helfre, G., Farjanel, J., Dubois, P., Fonvieille, J. and Frey, J. *Biochim. Biophys. Acta 429* (1976) 72.
6. Peterkofsky, B. and Assad, R. *J. Biol. Chem. 251* (1976) 4770.
7. Lehtinen, P., Vuorio, E. and Kulonen, E. *Biochem. J. 146* (1975) 565.
8. Bailey, A. J., Sims, T. J., Le Lous, M. and Bazin, S. *Biochem. Biophys. Res. Commun. 66* (1975) 1160.
9. Viljanto, J. and Kulonen, E. *Acta Pathol. Microbiol. Scand. 56* (1962) 120.
10. Lampiaho, K. and Kulonen, E. *Biochem. J. 105* (1967) 333.
11. Aalto, M., Lampiaho, K., Pikkarainen, J. and Kulonen, E. *Biochem. J. 132* (1973) 663.
12. Michell, R. H. and Hawthorne, J. N. *Biochem. Biophys. Res. Commun. 21* (1965) 333.
13. Gianetto, R. and de Duve, C. *Biochem. J. 59* (1955) 433.
14. Hübscher, G. and West, G. R. *Nature (London) 205* (1965) 799.
15. Swanson, P. D., Bradford, H. F. and McIlwain, H. *Biochem. J. 92* (1964) 235.
16. Hinton, R. H., Dobrota, M., Fitzsimons, J. T. R. and Reid, E. *Eur. J. Biochem. 12* (1970) 349.
17. Goldbarg, J. A. and Rutenburg, A. M. *Cancer 11* (1958) 283.
18. Earl, D. C. and Korner, A. *Biochem. J. 94* (1965) 721.
19. Martin, J. B. and Doty, D. M. *Anal. Chem. 21* (1949) 965.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem. 193* (1951) 265.
21. Schmidt, G. and Thannhauser, S. J. *J. Biol. Chem. 161* (1945) 83.
22. Bartlett, G. R. *J. Biol. Chem. 234* (1959) 466.
23. Svanborg, A. and Svennerholm, L. *Acta Med. Scand. 169* (1961) 43.
24. Burton, K. *Biochem. J. 62* (1956) 315.
25. Folch, J., Lees, M. and Sloane-Stanley, G. H. *J. Biol. Chem. 226* (1957) 497.
26. Badzio, T. and Doczon, H. *Clin. Chim. Acta 13* (1966) 794.
27. Pikkarainen, J. *Acta Physiol. Scand. Suppl. 309* (1968) 1.
28. Minari, O. and Zilversmit, D. B. *Anal. Biochem. 6* (1963) 320.
29. Juva, K. and Prockop, D. J. *Anal. Biochem. 15* (1966) 77.
30. Peterkofsky, B. and Diegelmann, R. E. *Biochemistry 10* (1971) 988.
31. Lehtinen, P. *Unpublished work.*
32. Harwood, R., Grant, M. E. and Jackson, D. S. *Biochem. J. 156* (1976) 81.

33. Ivaska, K. *Virchows Arch. B. 14* (1973) 19.
34. Kruse, N. J. and Bornstein, P. *J. Biol. Chem. 250* (1975) 4841.
35. Weinstock, M. and Leblond, C. P. *J. Cell Biol. 60* (1974) 92.
36. Olsen, B. R., Berg, R. A., Kishida, Y. and Prockop, D. J. *J. Cell Biol. 64* (1975) 340.
37. Nist, C., von der Mark, K., Hay, E. D., Olsen, B. R., Bornstein, P., Ross, R. and Dehm, P. *J. Cell Biol. 65* (1975) 75.
38. Dehm, P. and Prockop, D. J. *Biochim. Biophys. Acta 264* (1972) 375.
39. Schofield, D., Harwood, R. and Jackson, D. S. *Biochem. J. 166* (1977) 437.

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