

Radioautographic Characterization of Successive Compartments along the Rough Endoplasmic Reticulum-Golgi Pathway of Collagen Precursors in Foot Pad Fibroblasts of [³H]Proline-injected Rats

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ABSTRACT Young rats given an intravenous injection of [³H]proline were killed at successive times from 4 to 80 min later. Fibroblasts from the front foot pad were radioautographed; silver grains were counted over several of the organelles and the results were expressed as percent radiolabel per unit volume. These percentages reached a peak over rough endoplasmic reticulum cisternae at 4 min, intermediate vesicles and tubules at 10 min, spherical distensions of *cis*-side Golgi saccules at 20 min, cylindrical distensions of *trans*-side saccules between 40 and 60 min, and secretory granules at 60 min. It is proposed that the succession of peaks corresponds to the migration pathway of collagen precursor proteins within fibroblasts; that is, the proteins synthesized in rough endoplasmic reticulum are delivered by intermediate vesicles and/or tubules to the spherical distensions of *cis*-side saccules, somehow pass from there to the cylindrical distensions of *trans*-side saccules and, finally, are carried by secretory granules to the extracellular space.

The passage of collagen precursors through the Golgi apparatus of collagen-producing cells was initially denied (1–3), but later demonstrated by direct identification of procollagen (4, 5) and also by procollagen immunostaining (6–8) and [³H]-proline radioautography (9–14). By the last method, peaks of silver grain counts were successively observed over rough endoplasmic reticulum (rER),¹ Golgi apparatus, and secretory granules. It was further reported that in Golgi stacks, silver grains first appeared over the spherical distensions found along *cis*-side saccules and later over cylindrical distensions on *trans*-side saccules (9, 14), but these observations were not quantitated. In view of present interest in Golgi compartmentation (15–20), we decided to quantitate the radioautographic reactions observed over fibroblast organelles at various times after [³H]proline injection with particular attention to the spherical and cylindrical distensions of Golgi saccules.

MATERIALS AND METHODS

Use was made of material prepared in a previous investigation of foot pad fibroblasts from 20-d-old rats killed at various times after an intravenous

injection of 2.5 mCi of L-[2,3-³H]proline (14). The radioautographs were developed by gold thiocyanate latensification followed by Alfa-Gevaert solution physical developer (21). This procedure improves resolution since the "half distance" (distance from a radioactive line source within which 50% of the silver grains fall, reference 22) is 76 nm in Ilford L-4 emulsion instead of 187 nm after filamentous grain development (23), probably because each silver grain is located within the space previously occupied by the activated silver bromide crystal (21). The silver grain, which may consist of one, two, or more spherical silver deposits (21), is identified by placing over it a transparent plastic sheet on which is drawn a circle with a 5-mm diam (corresponding to the 140 nm diam of a silver bromide crystal at a magnification of 36,000). If a silver deposit is located in such a way that no other can be included within the circle, it is considered as one grain. If two or more silver deposits can be included in the circle, they are collectively considered as one grain. Preliminary counts revealed that the conditions were suitable for direct scoring (24); that is, each grain could be considered as arising from the cytoplasmic structure subjacent to the center of the circle.

Using 18 to over 30 radioautographs of fibroblasts at × 36,000 for each time interval, silver grains were assigned to one of the previously described compartments (14) identified as follows: (a) "rER cisternae," comprising cisternal membrane and content, as well as the associated ribosomes; (b) "intermediate elements," comprising the intermediate vesicles and tubules located between rER cisternae and the *cis* face of Golgi stacks (Fig. 1); (c) "spherical distensions" of first and second saccules characterized by a circular or oval profile and a content of coiled threads (Figs. 1 and 2); (d) "cylindrical distensions" of the next three saccules characterized by a more or less rectangular profile and a content of aligned threads (Figs. 1 and 2) distinguishable in longitudinal and

¹ Abbreviation used in this paper: rER, rough endoplasmic reticulum.

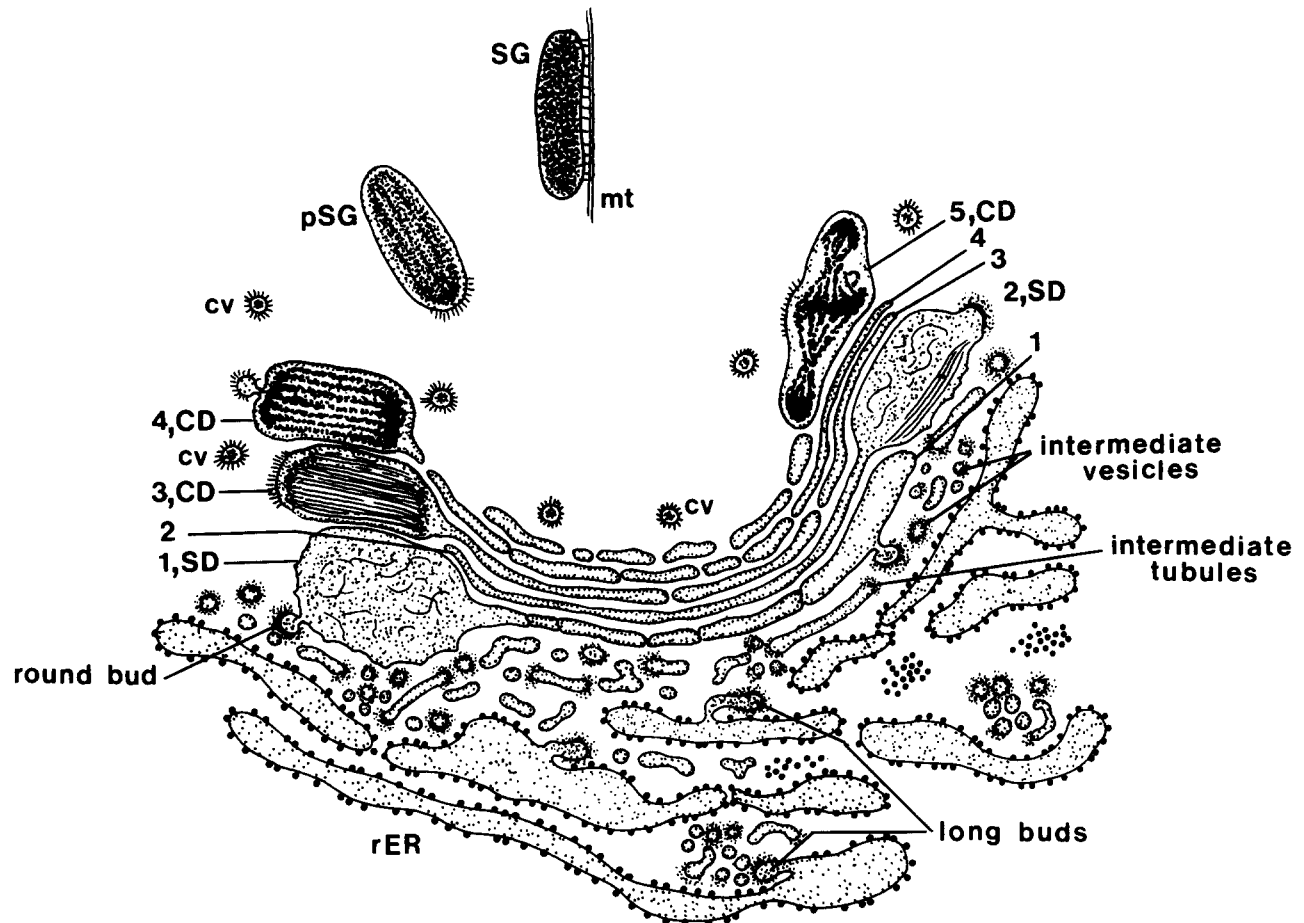


FIGURE 1 Diagram of a stack of Golgi saccules and associated rER from a foot pad fibroblast of a 20-d-old rat. On the side facing the Golgi stack, some rER cisternae carry "long buds" free of ribosomes and coated with fuzzy material. Next, there are intermediate elements of two types: vesicles frequently coated with fuzzy material and tubules whose extremities show similar fuzzy material. The first saccule on the *cis* face of the Golgi stack is fenestrated and includes large spherical distensions (1,SD) containing looping threads. Round buds coated with fuzzy material may be seen along saccule and distension. The second saccule is usually not fenestrated and includes spherical distensions, but their entangled threads may be associated with a few parallel ones (2,SD). The third saccule shows cylindrical distensions which contain fine parallel threads (3,CD) and carry typical coated buds and patches, while typical coated vesicles (cv) are seen nearby. The fourth saccule also includes cylindrical distensions containing parallel threads (4,CD), but these appear thick as a result of aggregation of fine threads (14). The last saccule is short, fenestrated, and again associated with cylindrical distensions containing thick parallel threads, which are occasionally twisted (5,CD). Beyond the *trans* face, prosecretory granules (pSG) contain barely visible threads, while secretory granules (SG) have a homogeneous content; they are joined by tiny processes to microtubules (mt).

TABLE I
Radioactivity of Cytoplasmic Compartments of Foot Pad Fibroblasts at Various Times after [³H]Proline Injection*

	Time after [³ H]proline injection					
	4 min	10 min	20 min	40 min	60 min	80 min
rER cisternae	71.7	63.2	46.7	27.4	29.9	36.8
Intermediate elements	11.5	19.0	14.1	11.3	10.1	14.5
Spherical distensions	3.7	4.9	10.7	8.6	6.9	2.9
Cylindrical distensions	0	1.2	13.3	33.1	34.2	29.8
Flat Golgi saccules	3.7	1.8	6.9	9.7	3.1	5.4
Secretory granules	0	0	1.5	5.1	9.9	6.2
Other structures	9.4	9.8	6.8	4.7	5.9	4.6
No. of photographs	34	26	21	21	18	19
Mean no. of silver grains per photograph	5.7	6.3	25.4	39.2	66.3	12.7

* Expressed as percentage of intracellular silver grains. The highest percentages observed for any structure are italicized.

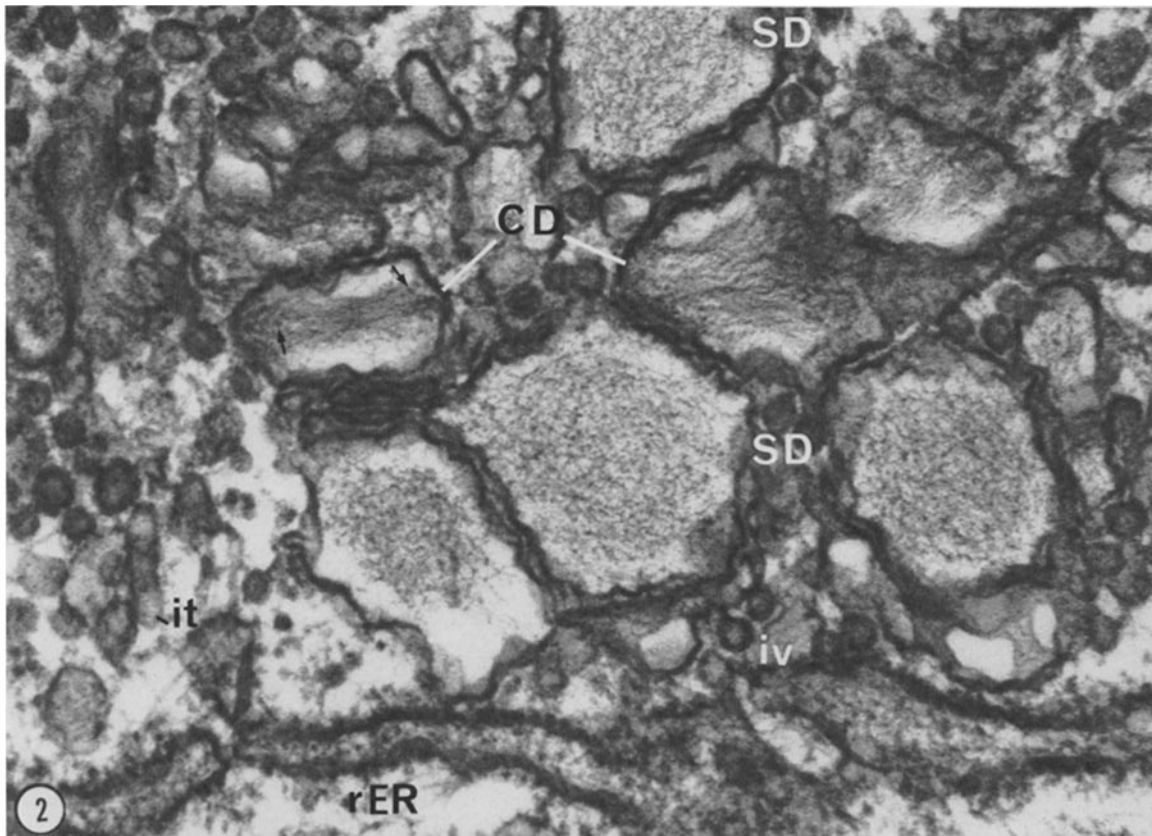


FIGURE 2 Golgi region of foot pad fibroblast after fixation in reduced osmium. The Golgi apparatus is cut along its edge, but the distensions and their content are visible. A row of rER cisternae occupies the base of the figure (rER). Above, intermediate vesicles (iv) are scattered as are an intermediate tubule at left (it) and, toward the center, three spherical distensions (SD) containing irregular looping threads. Higher up, cylindrical distensions may be seen (CD); in the one at center right, threads are beginning to line up whereas in the one at left, the alignment is completed. Each thread is believed to be composed of single or aggregated procollagen molecules, whose propeptides are indicated by small arrows. $\times 77,000$.

cross section; (e) "flat Golgi saccules," consisting of the nondistended portions; (f) "secretory granules" including prosecretory and secretory granules, and (g) "other structures" including intervening spaces between organelles as well as nucleus, mitochondria, dense and multivesicular bodies, etc. (Table I).

To obtain an index of the volume of the organelles under study, we applied the point hit method (25) to each radioautograph immediately after silver grains had been enumerated. Over 10,000 point hits were recorded in 139 micrographs (Table II). Silver grains were also counted over fibroblasts in light microscopic radioautographs of 1- μm -thick sections at $\times 1,000$ (Table III).

RESULTS

The radioactivity present in the organelles of fibroblasts² was

² The following experiments were designed to find out how much of the injected [³H]proline was incorporated within the collagenous proteins of foot pad tissues. Epon blocks of foot pad were prepared first from one animal killed at 60 min and secondly from a series of animals killed at 20, 40, or 80 min or 24 h after [³H]proline injection. The blocks were cut into 1- μm -thick sections which were then hydrolyzed in 6 N hydrochloric acid for 24 h under nitrogen. The proline and hydroxyproline fractions were obtained from an amino acid analyzer and their radioactivity was determined. The proportion of label in collagen and noncollagenous protein was then calculated (26, 27), using a 1.31 ratio of proline to hydroxyproline for collagen.

The foot pad of the 60-min animal showed 56.7% of the label in collagen. In the later series, the percent of label in collagen was 25.3 and 27.8% at 20 min, 27.4, and 29.7% at 40 min, 43.3, 44.7, and 52.4% at 80 min, and 57.2% at 24 h. It was concluded that except at 20 and 40 min, about half of the labeled proline was in collagen or,

initially expressed as percentage of the intracellular silver grains (Table I). At 4 min after [³H]proline injection, the percentage over rER cisternae was higher than over other organelles, but at later times it decreased gradually (except at 80 min when much of the radioactivity had left the cells, Table III). The percentage over intermediate elements increased from 4 to 10 min and decreased later. The percentage over spherical Golgi distensions also increased with time, reached a high at 20 min, and decreased thereafter. Over the cylindrical Golgi distensions, labeling started at the 10-min interval and the percentage increased to high figures at 40 and 60 min, with the peak presumably occurring between these two times. The flat portion of Golgi saccules showed somewhat irregular variation, the highest values being at 20 and 40 min. In secretory granules, labeling first appeared at 20 min and the percentage increased to its highest value at 60 min. The values for "other structures" did not seem to show a significant trend.

more precisely, in the helical portion of fibroblast procollagen and extracellular collagen. The other half was assumed to be mainly in proteins elaborated by the cells of sweat glands, muscle, blood vessels, and other structures present in the foot pad, although some label could also be in noncollagenous proteins produced by fibroblasts, e.g., fibronectin. The smaller percent of label in collagenous proteins at 20 and 40 min could be explained by incomplete hydroxylation of pro- α chains at these times.

The percentage of silver grains (Table I) was divided by the percentage of point hits (indicative of relative volume, Table II) to obtain the percentage of radiolabel per unit volume. The results (Fig. 3) showed a sequence of peaks: in rER cisternae at 4 min, intermediate elements at 10 min, spherical distensions at 20 min, and cylindrical distensions between 40 and 60 min. In the secretory granules, which were left out of Fig. 3 for the sake of clarity, the percentage of radiolabel per unit volume was 1.3, 4.4, 8.5, and 5.3 respectively at 20, 40, 60, and 80 min; therefore, a labeling peak was reached at 60 min.

DISCUSSION

When a radiolabeled substance migrates from compartment to compartment, peaks of specific activity occur in sequence in the successive compartments (28) as do peaks of radiolabel

TABLE II
Percentage of Point Hits in Cytoplasmic Compartments of Foot Pad Fibroblasts*

	Number of hits %
rER cisternae	51.22
Intermediate elements	9.99
Spherical distensions	4.76
Cylindrical distensions	12.00
Flat Golgi saccules	6.23
Secretory granules	1.17
Other structures	14.62

* Hits were recorded in all micrographs in which silver grains were observed. A total of 10,057 was registered.

TABLE III
Light Microscopic Counts of Silver Grains Per Unit Area over Foot Pad Fibroblasts at Various Times after [³H]Proline Injection

	4 min	10 min	20 min	40 min	60 min	80 min
Number of cells examined	13	13	10	10	6	6
Mean grain count minus background	16.1	19.6	55.3	79.4	77.0	12.9

content. Thus, the sequence of peaks is indicative of the pathway followed by the label. Because of individual variation, the results are not expressed as radiolabel content but as percentage of the total radiolabel present in the cells (Table I and Fig. 3). It is shown in the Appendix that in such a case, the sequence of peaks is reliable only at times when the total radiolabel content is at or near its maximum. Therefore, the total radiolabel content of fibroblasts was measured (Table III) and found to approximate a plateau from 20 to 60 min. We concluded that in Fig. 3, the timing was reliable in the case of the 20-min peak over spherical distensions, the 40–60-min peak over cylindrical distensions, and the 60-min peak over secretory granules. In the other cases, the true peak of radiolabel content occurred earlier than the recorded times, that is, before 4 min for rER cisternae and before 10 min for intermediate elements.

The initial peak over rER cisternae (Fig. 3) was attributed to [³H]proline incorporation into the pro-alpha chain precursors of collagen (29–31); it probably occurred as soon as the incorporation began. The peak over intermediate elements, which probably took place before the 10-min interval, was attributed to the transport of collagen precursors from rER to Golgi apparatus by intermediate vesicles and tubules, in accordance with earlier proposals (32–35). The 20-min peak over spherical distensions was attributed to labeling of their content of looping threads, whereas the peak reached between 40 and 60 min over cylindrical distensions was attributed to labeling of their aligned threads, previously identified as procollagen (4, 5). The sequence indicated that the looping threads straightened out into the aligned threads and were presumably at a last stage in the transformation of aggregated pro-alpha chains into procollagen. Finally, the 60-min peak in secretory granules (not shown in Fig. 3) confirmed that they arose from cylindrical distensions that had been freed from their saccule (14).

In conclusion, the results provide a demonstration of two previously suspected steps in the biosynthetic pathway of type I collagen: transport of precursors by intermediate elements from rER to Golgi spherical distensions and transformation of spherical into cylindrical distensions. How this transformation is associated with a migration from *cis* to *trans* side is not clear. The simplest explanation is that whole saccules

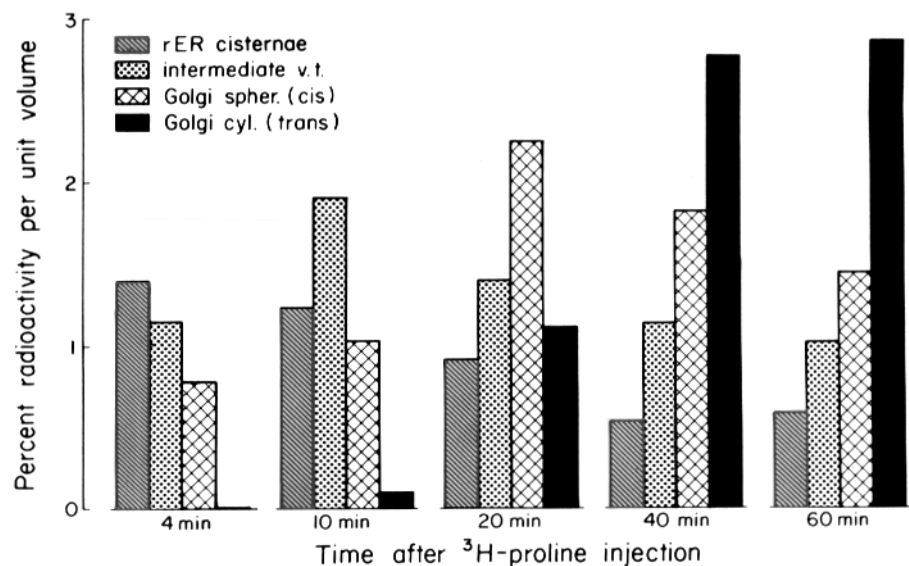


FIGURE 3 Percent radioactivity per unit volume, estimated in four fibroblast compartments from 4 to 60 min after injection of [³H]proline. Peaks are observed in rER cisternae at 4 min, intermediate vesicles and tubules at 10 min, Golgi spherical distensions of *cis*-side saccules at 20 min, and Golgi cylindrical distensions of *trans*-side saccules at 40–60 min.

including distensions migrate from the *cis* side of the stack where they are formed to the *trans* side where they give rise to secretory granules. Such a hypothesis has been proposed for mucous cells (36), but has been rejected on the basis of experiments with other cell types (37).

APPENDIX

Norman J. Nadler

Assume that in certain cells, z structures are recognized as radiolabeled and that the radioactivity content of each structure expressed as a function of time is $p_1(t), p_2(t) \dots p_n(t) \dots p_z(t)$, while the sum of these is $\sum_{n=1}^{n=z} p_n(t)$. For the sake of brevity, $p(t)$ will be represented by p and $\sum_{n=1}^{n=z} p_n(t)$ by Σ .

The ratio of radioactivity per structure (referred to for each structure in Table I as percentage of silver grains in foot pad fibroblasts) is p/Σ . The first derivative at any given time is

$$\frac{d}{dt} \frac{p}{\Sigma} = \frac{\Sigma \frac{d}{dt} p - p \frac{d}{dt} \Sigma}{\Sigma^2}$$

Dividing by p/Σ ,

$$\frac{\frac{d}{dt} \frac{p}{\Sigma}}{\frac{p}{\Sigma}} = \frac{1}{p} \frac{dp}{dt} - \frac{1}{\Sigma} \frac{d\Sigma}{dt}$$

At time t_{\max} when, for any structure, p/Σ reaches a maximum, then $(d/dt)(p/\Sigma) = 0$ and therefore

$$\frac{1}{p} \frac{dp}{dt} = \frac{1}{\Sigma} \frac{d\Sigma}{dt}$$

This means that at time t_{\max} , dp/dt has the same sign as $d\Sigma/dt$, so that
 (a) if $d\Sigma/dt > 0$, i.e., Σ is rising, p is also increasing and therefore reaches a peak later than p/Σ ;
 (b) if $d\Sigma/dt < 0$, i.e., Σ is decreasing, p is also decreasing and therefore reaches a peak earlier than p/Σ ; and
 (c) if $d\Sigma/dt = 0$, i.e., Σ is at a maximum, p/Σ is also at a maximum.

Hence, for any given structure, n , only if the time when the ratio p_n/Σ is at a maximum falls in the time range when Σ is maximal will the peak for the ratio p_n/Σ correspond with the peak for p_n . Otherwise, if the peak for the ratio p_n/Σ precedes the time range when Σ is maximal, so does the peak for the ratio p_n/Σ also precede the peak for p_n ; and, if the peak for the ratio p_n/Σ occurs after the time range when Σ is maximal, so does the peak for the ratio p_n/Σ occur after the peak for p_n .

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