

RADIOAUTOGRAPHIC STUDY OF IN VIVO AND IN VITRO INCORPORATION OF FUCOSE-³H INTO THYROGLOBULIN BY RAT THYROID FOLLICULAR CELLS

A. HADDAD, MEREDITH D. SMITH, ANNETTE HERSCOVICS,
N. J. NADLER, and C. P. LEBLOND

From the Department of Anatomy, McGill University, Montreal, Canada

ABSTRACT

The incorporation of fucose-³H in rat thyroid follicles was studied by radioautography in the light and electron microscopes to determine the site of fucose incorporation into the carbohydrate side chains of thyroglobulin, and to follow the migration of thyroglobulin once it had been labeled with fucose-³H. Radioautographs were examined quantitatively in vivo at several times after injection of fucose-³H into rats, and in vitro following pulse-labeling of thyroid lobes in medium containing fucose-³H. At 3–5 min following fucose-³H administration in vivo, 85% of the silver grains were localized over the Golgi apparatus of thyroid follicular cells. By 20 min, silver grains appeared over apical vesicles, and by 1 hr over the colloid. At 4 hr, nearly all of the silver grains had migrated out of the cells into the colloid. Analysis of the changes in concentration of label with time showed that radioactivity over the Golgi apparatus increased for about 20 min and then decreased, while that over apical vesicles increased to reach a maximum at 35 min. Later, the concentration of label over the apical vesicles decreased, while that over the colloid increased. Similar results were obtained in vitro. It is concluded that fucose, which is located at the end of some of the carbohydrate side chains, is incorporated into thyroglobulin within the Golgi apparatus of thyroid follicular cells, thereby indicating that some of these side chains are completed there. Furthermore, the kinetic analysis demonstrates that apical vesicles are the secretion granules which transport thyroglobulin from the Golgi apparatus to the apex of the cell and release it into the colloid.

INTRODUCTION

The polypeptide components of thyroglobulin, like those of other glycoproteins, are synthesized in relation with ribosomes (Nadler et al., 1964). After their completion, carbohydrate side chains of two types are added, one type consisting of *N*-acetylglucosamine and mannose, and the other of an inner core of the same two monosaccharides to which are attached branches beginning with *N*-acetylglucosamine, continuing with galactose, and terminating with sialic acid or fucose (Spiro

and Spiro, 1965; Spiro, 1965; Arima et al., 1970). There is evidence that these sugars are added in a stepwise manner to the polypeptide chains as they migrate through the thyroid follicular cell on their way to the colloid lumen (Spiro and Spiro, 1966; Herscovics, 1969; Whur et al., 1969). In particular, radioautographic studies making use of thyroid lobes in vitro have demonstrated that mannose is taken up in the rough endoplasmic reticulum, whereas galactose is incorporated in the Golgi

apparatus (Whur et al., 1969). The uptake of galactose in the Golgi apparatus has been recently confirmed *in vivo* by using an improved technique, in which nonradioactive galactose is added to the fixation fluid (Haddad, 1971).

Since the position of fucose in the thyroglobulin molecule is at the end of some carbohydrate side chains, it was of interest to find out whether this sugar is also taken up in the Golgi apparatus or is incorporated elsewhere, perhaps at some later stage in the migration of thyroglobulin towards the colloid lumen. Biochemical work had already shown that the label from fucose is incorporated as such into glycoproteins (Coffey et al., 1964; Bekesi and Winzler, 1967; Kaufman and Ginsburg, 1968) and particularly into thyroglobulin (Herscovics, 1970). It should, therefore, be possible by means of radioautography to make a precise identification of the organelle in which fucose label is taken up into thyroglobulin. Furthermore, once thyroglobulin is labeled with fucose-³H, it should be possible to trace the pathway of its migration toward the colloid. These two aspects of the problem were examined *in vivo* by injecting fucose-³H into rats which were killed at various times thereafter, and *in vitro* by incubating thyroid lobes in a medium containing fucose-³H.

MATERIALS AND METHODS

In Vivo Experiments

Sherman male rats weighing about 40 g each were injected intravenously with 5 mCi of L-fucose-³H (G) (SA 4.3 Ci/mole, New England Nuclear Corp., Boston, Mass.; 0.19 mg in 5.00 ml of 90% ethanol). In a first step, the original solution of fucose-³H in 90% ethanol was evaporated to dryness in a Flash Evaporator (Buchler Instruments, Inc., Fort Lee, N. J.). Then 1–2 ml of distilled water was added and a second evaporation was performed until a volume adequate for intravenous injection was reached (0.15 ml/animal). The animals were killed 3, 5, 20, and 35 min, 1, 4, and 30 hr after the injection, by perfusing fixative through the left ventricle. The fixative used in these experiments had the following composition: 2.5% glutaraldehyde (TAAB Laboratories, Reading, England) in 0.05 M Sorensen's phosphate buffer containing 0.1% sucrose and 0.25% nonradioactive L-fucose (433 milliosmolar). After 12 min of perfusion at room temperature, the thyroid gland was removed and immersed in the same fixative for 1½ hr at 4°C. The tissue was then washed in 0.1 M Sorensen's phosphate buffer at 4°C, during which time the thyroid glands were chopped into small pieces. These were postfixed in

1% osmium tetroxide in 0.1 M Sorensen's phosphate buffer for 2 hr at 4°C, dehydrated in ethanol, and, through propylene oxide, embedded in Epon.

1- μ thick sections were mounted on glass slides and coated with NTB2 nuclear emulsion (Eastman Kodak, Rochester, N.Y.). After suitable exposure, radioautographs were developed in Kodak D-170 (Baserga and Malamud, 1969). The radioautographs were stained with 1% toluidine blue and examined in the light microscope.

Thin sections showing silver interference color were prepared for electron microscope radioautography as described previously (Whur et al., 1969).

GRAIN COUNTS: Grain counts were carried out in light and electron microscope radioautographs.

For *light microscopy*, 0.5 μ semi-thin sections from thyroids obtained at all time intervals were mounted on the same slide and exposed for 10 days. A Zeiss micrometer was inserted into a microscope with a $\times 10$ ocular and a $\times 100$ objective; each one of its squares covered an area of 100 μ^2 . The section was moved so that the limit between two squares was over the border between a cell and the colloid. Under these conditions, one of the squares covered the supranuclear region and the other was over the adjacent area of colloid. The number of silver grains was then counted in both squares. After this was done over 60 cells/section, the results were expressed as an average concentration of silver grains per 110 μ^2 at each time interval (Table I). Since the number of grains at the base of the cells and at the center of the colloid was negligible at all time intervals, these data represent an estimate of the relative total radioactivity per cell (and adjacent colloid) at the different times.

TABLE I
Grain Counts over Thyroid Cell Apex and Adjacent Colloid in Light Microscope Radioautographs after Injection of Fucose-³H in Vivo

Time after injection	Number of silver grains over 55 μ^2 of cell apex and the adjacent 55 μ^2 of colloid*
3–5 min	2.9 \pm 1.2
20 min	8.1 \pm 0.7
35 min	9.0 \pm 1.6
1 hr	9.2 \pm 1.3
4 hr	12.0 \pm 1.9

* Since the numbers of grains over the cell base and over the center of the colloid were negligible at all times, these results are an index of the amount of labeled thyroglobulin in relation to a given volume of cell.

TABLE II
Crude Counts over the Supranuclear Region of Follicular Cells Following Injection of
Fucose-³H *in Vivo*

Time after intravenous injection	3-5 min	20 min	35 min	1 hr	4 hr
Exclusive grains					
Golgi	257	140	37	25	8
rER	29	43	20	18	6
Apical vesicles	0	24	46	30	30
Colloid	0	0	15	85	493
Nucleus	4	2	0	0	8
Total	290	209	118	158	545
Shared grains					
Golgi + others*	0	4	3	2	0
rER + others*	13	54	28	27	12
Apical vesicles + others*	0	2	15	14	8
Colloid + others*	0	0	0	0	0
Golgi + rER	114	112	37	10	4
Golgi + rER + others*	16	6	1	0	0
Apical vesicles + rER	0	73	82	47	26
Apical vesicles + rER + others*	0	10	19	5	0
Apical vesicles + Golgi	9	0	11	10	0
Apical vesicles + Golgi + others*	0	0	0	0	0
Apical vesicles + Colloid	0	0	12	26	57
Apical vesicles + Colloid + others*	0	0	0	0	0
Total	152	261	208	141	107
Grand total	442	470	326	299	652

* "Others" refers to those structures to which no exclusive grains were found to be assigned; they include mitochondria, lysosomes, colloid droplets, and plasma membrane.

TABLE III
Per Cent Hits on Several Follicular Cell Structures (and Colloid) using the "Point Hit"
and "Circle Hit" Methods

Time after injection	Golgi	Rough ER	Apical vesicles	Microvilli (and adjacent colloid)	Colloid
3-5 min					
Point hits	9.7	54.9	3.7	3.2	
Circle hits	10.0	51.1	5.6	3.2	
20 min					
Point hits	9.1	50.7	6.2	5.9	
Circle hits	9.5	47.1	7.8	5.1	
35 min					
Point hits	8.7	45.3	8.2	8.3	
Circle hits	8.0	43.2	11.7	6.7	
1 hr					
Point hits	6.2	48.1	7.1	10.5	
Circle hits	6.0	43.5	8.8	9.7	
4 hr					
Point hits	3.2	38.2	8.9	12.5	12.0
Circle hits	3.6	37.4	11.2	12.2	5.7

Grain counts were also carried out in *electron microscope* radioautographs of thyroid cells at all time intervals up to and including 4 hr. At each time interval, radioautographs of about 45 thyroid follicular cells were photographed at $\times 30,000$. In some cases, sections of whole follicular cells were included in the picture. When this was not possible, the area containing the highest concentration of silver grains was photographed. From 300 to 600 silver grains were examined at each time interval. Each grain was circumscribed within a "resolution boundary circle" of 6.7 μ m radius by methods described previously (Whur et al., 1969). This "resolution boundary circle" has been calculated to contain the source of radioactivity giving rise to the silver grain with 95% probability.¹ The organelle(s) present within the circles were recorded, and the cases in which a single organelle was within a circle ("exclusive" grains) were distinguished from those cases in which a circle encompassed two or more organelles ("shared" grains). These crude grain counts are recorded in Table II.

The method described in the Appendix by Nadler has been used to assign the shared grains to only one class of structures, thus giving the corrected grain counts for each organelle which are recorded in Fig. 21 in the Discussion section as per cent of the total number of grains. For these calculations of the corrected grain counts, the distribution of each organelle was assessed by both the "point hit method" and the "circle hit method" (see Appendix), and expressed as percentage of the total number of points or circles used (Table III). The differences at the various time intervals are due to the fact that the photographs were centered on the silver grains, which were nearer the apical cell surface as time went on. The relative concentration of grains per organelle at each time interval was obtained by dividing the per cent corrected grain count by the per cent volume obtained by the point hit method (Fig. 22). Finally, the changes in concentration of radioactivity per organelle with time were calculated by multiplying the concentration of grains per organelle by the total radioactivity per cell (and adjacent colloid) at each time interval as estimated in Table I. These results are reported in graph form (Fig. 23).

In Vitro Experiments

Sherman male rats weighing about 60 g each were anesthetized with ether, and the larynx and part of the trachea bearing the thyroid gland were removed and immediately placed in previously oxygenated

¹ Nadler, N. J. 1971. Estimation of resolving power in electron microscope radioautography. Manuscript in preparation.

saline at 4°C. The thyroid lobes were isolated by dissection within the saline.

A modification of the technique described by Jensen et al. (1964) was used for incubation of the lobes. A strip of filter paper 1.5 \times 5.0 cm (Whatman No. 40) was placed over a stainless steel raft with its extremities folded under the raft. Holes were made in the filter paper to accommodate the thyroid lobes. The raft with the filter paper was placed inside a small Petri dish (35 \times 10 mm) which contained 2 ml of medium. The medium did not reach the top of the raft, but the edges of the filter paper were dipping into the medium. All incubations were performed with the Petri dish inside a desiccator kept in an oven at 37°C. There was a continuous flow of a humidified gas mixture (95% O₂:5% CO₂) in the desiccator. The filter paper and the thyroid lobes could remain wet for several hours

TABLE IV
Crude Counts over the Supranuclear Region of Follicular Cells Following Incubation with Fucose-³H in Vitro

Total incubation time*	15 min	30 min	135 min
Exclusive grains			
Golgi	318	78	8
rER	117	64	31
Apical vesicles	7	57	14
Colloid	3	52	136
Mitochondria	3	10	3
Others	11	15	12
Total	459	276	196
Shared grains‡			
Golgi	49	21	2
rER	79	65	22
Apical vesicles	4	35	21
Colloid	0	14	11
Mitochondria	26	39	14
Others	20	18	5
Total	178	192	75
Grand total	637	468	271

* The lobes were pulse-labeled for 15 min in medium containing fucose-³H and then transferred to fresh unlabeled medium for the rest of the incubation time.

‡ The shared grains were quantitated by the simplified method used by Whur et al. (1969), that is, when a grain was shared by a number of structures, X, a value 1/X was assigned to each structure. The counts shown in this table represent the total of such values assigned to each structure.

without requiring the addition of fresh medium to the Petri dish.

At the beginning of the incubation, the thyroid lobes were dipped in prewarmed and pre-gassed bicarbonate-buffered Krebs-Ringer solution containing 500 $\mu\text{Ci/ml}$ of L-fucose- ^3H with a specific activity of 4.3 Ci/mole (pulse medium) and then placed on the filter paper wetted with the same medium. One thyroid lobe was removed from the pulse medium 5 min after incubation and processed for radioautography as indicated below. The other lobes remained in the pulse medium for 15 min. One lobe was removed at the end of this time for processing. The others were washed by immersing the raft with the filter paper bearing the lobes in Medium 199 with Earle's base (Baltimore Biological Laboratory, Baltimore, Md.) containing 10 mM of nonradioactive L-fucose ("chase" medium). Then the raft was transferred to a Petri dish containing 2 ml of fresh "chase" medium. The thyroid lobes were incubated in this medium for 15, 30, and 45 min, 1, 2, 4, and 6 hr.

After incubation, the thyroid lobes were fixed by immersion in 4% paraformaldehyde in 0.1 M Sorensen's phosphate buffer for 1½ hr at 4°C. Then they were washed in 0.15 M Sorensen's buffer, postfixated in osmium tetroxide, and embedded in Epon. These thyroid lobes were processed for electron microscope radioautography and examined as indicated for *in vivo* experiments. In addition, in some other experiments, the lobes were fixed in Bouin's, embedded in paraffin and processed for light microscope radioautography.

GRAIN COUNTS: Grain counts were carried

out in electron microscope radioautographs by using the simplified method described by Whur et al. (1969). The crude grain counts are reported in Table IV, and the relative concentration of label, in the Discussion section (Fig. 24).

RESULTS

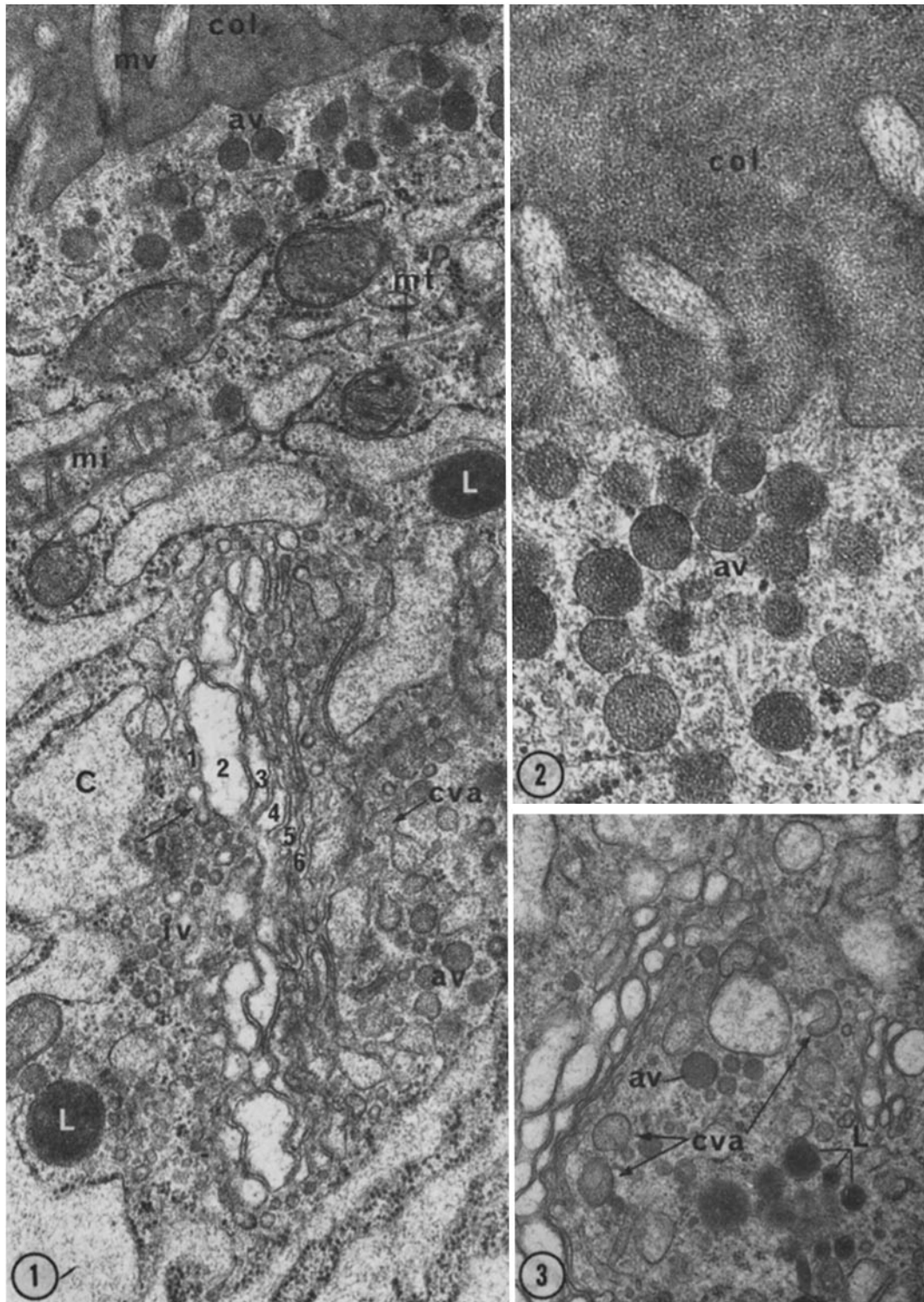
The follicular cell (Fig. 1) is in contact with the colloid which occupies the lumen of the thyroid follicle. The apical surface of the cell shows numerous microvilli. Below this surface, an array of dark circular profiles identifies the apical vesicles. The rest of the cytoplasm is packed with distended cisternae of rough endoplasmic reticulum and a fair number of mitochondria; in addition, there are a few dense bodies (presumed to be lysosomes) and some microtubules. These various structures have often been described in the literature (Wissig, 1960; Nadler et al., 1964; Lupulescu and Petrovici, 1968). However, it was felt that a detailed description of the Golgi apparatus of the follicular cell might help in understanding the formation of thyroglobulin.

The Golgi apparatus depicted in the lower half of Fig. 1 will serve as a model. While the cisternae of rough endoplasmic reticulum located at a distance from the Golgi apparatus show a fairly regular array of ribosomes along their surfaces, those cisternae located next to the Golgi apparatus show few or no ribosomes on the side facing the

FIGURE 1 Electron micrograph of a portion of a thyroid follicular cell. This and subsequent electron micrographs (except Fig. 10) were stained with uranyl acetate and lead citrate. At the top, microvilli (*mv*) extend from the apical cell border into the colloid (*col*). Below the border, apical vesicles (*av*) with gray content are prominent. Deeper still, mitochondria (*mi*) alternate with cisternae of endoplasmic reticulum (*C*); a few microtubules (*mt*) and the odd dense body, presumed to be lysosomal in nature (*L*), may also be seen. In the Golgi region, the endoplasmic reticulum cisternae show few ribosomes on the side facing the Golgi apparatus, as shown at *C*. Small intermediate vesicles (*iv*) are often interspersed between such a cisterna and Golgi saccules. At the arrow, some of these vesicles appear to be fusing to give rise to the outermost saccule (indicated by the number 1). The second saccule (2) in the Golgi stack is distended, while subsequent saccules become increasingly flattened (3-6). Irregularly shaped condensing vacuoles (*cva*) are present nearby, as well as apical vesicles (*av*) similar to those seen below the apical cell borders. $\times 35,000$.

FIGURE 2 Electron micrograph of the apical cell surface of a thyroid follicular cell. Lettering is the same as in Fig. 1. The content of apical vesicles (*av*) shows gray threads and light areas. This pattern is clearly seen at lower left. A similar pattern is observed within the colloid (*col*). $\times 55,000$.

FIGURE 3 Electron micrograph of the Golgi region of a thyroid follicular cell. The stack of saccules is at left. A number of condensing vacuoles are seen, some of which have an irregular shape (*cva*). These may be compared with a nearby apical vesicle (*av*) and dense bodies (*L*). $\times 30,000$.



Golgi apparatus; thus, the cisterna labeled *C* in Fig. 1 has only a few ribosomes on this side. Between this cisterna and the stack of Golgi saccules, groups of "intermediate" vesicles may be seen (*iv* in Fig. 1), which are rather small (500–600 Å) and are often coated with a fine-textured fuzz. Some of them appear to fuse (arrow in Fig. 1) to give rise to the outermost or first saccule of the stack (*1* in Fig. 1).

The Golgi stack is usually composed of five to seven saccules. The first saccule is often incomplete and irregular, and is fenestrated. The second saccule is usually characterized by distension and the absence of distinguishable content (*2* in Fig. 1). The next few saccules show a gradual collapse with the appearance of a grayish content (*3, 4, 5* in Fig. 1). Finally, the last or innermost saccule (*6* in Fig. 1) is rather variable and seems to give rise to irregular spheroids with a fairly dense content, which, by comparison with the pancreas are referred to as condensing vacuoles (*cva* in Figs. 1 and 3). Transitions are seen between these and darker vesicles which are identical to the vesicles found at the apex of the cells and will also be referred to as apical vesicles (*av* in Figs. 1 and 3). These, which measure around 1700 Å in diameter, have a more regular, round outline and a denser content than condensing vacuoles. In addition, large vesicles (around 1200 Å in diameter) coated with distinct bristles are often present.

When the apical vesicles are examined under high power, they display an inner structure consisting of uniform gray threads alternating with light areas in a characteristic pattern (Fig. 1). This pattern, which is emphasized in Fig. 2 by a

slight overfocusing, is regularly found after glutaraldehyde fixation. The luminal colloid, when examined under high power, shows the same pattern of gray threads and light areas as the content of apical vesicles (Fig. 2).

For the presentation of both *in vivo* and *in vitro* experiments, the sequence of events will be reported first in the light microscope and then in the electron microscope; grain counts will be presented last.

In Vivo Experiments

LIGHT MICROSCOPE RADIOAUTOGRAPHY: At 3 and 5 min after injection of labeled fucose, silver grains were concentrated over a small area next to the nucleus of thyroid follicular cells (Fig. 4). At 20 min, some grains also appeared over the cell apex. By 35 min, the concentration of silver grains next to the nucleus was decreased and that over the apex was increased (Fig. 5). At 1 hr, silver grains were mainly over the apex, with some also appearing over the colloid (Fig. 6). By 4 hr, most grains were over the colloid, although they remained close to the cell apex and thus formed well-defined rings (Fig. 7). Finally, at 30 hr the silver grains were spread more widely over the colloid and there were hardly any grains

ELECTRON MICROSCOPE RADIOAUTOGRAPHY: At 3 and 5 min after fucose-³H injection, the silver grains were almost exclusively over the Golgi apparatus of follicular cells (Fig. 9). Most Golgi grains were over the stacks of saccules, without apparent preference for any one saccule. A few silver grains seemed to be over intermediate vesicles, and very few were over nearby cisternae

FIGURES 4–8 Light microscope radioautographs of rat thyroid lobes at various time intervals after injection of fucose-³H *in vivo*. Stained with toluidine blue after radioautography. Each figure shows one or more thyroid follicles containing a central colloid area surrounded by follicular cells. $\times 625$.

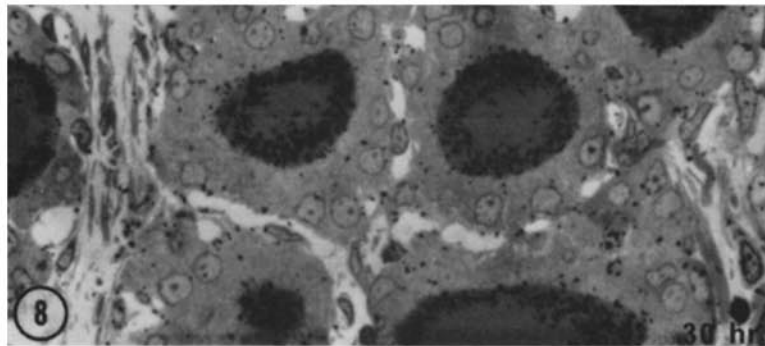
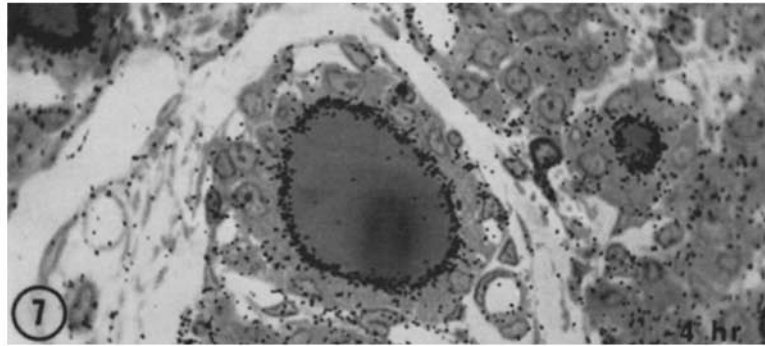
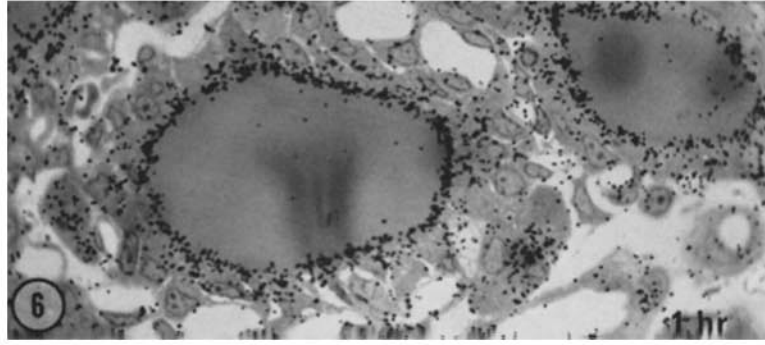
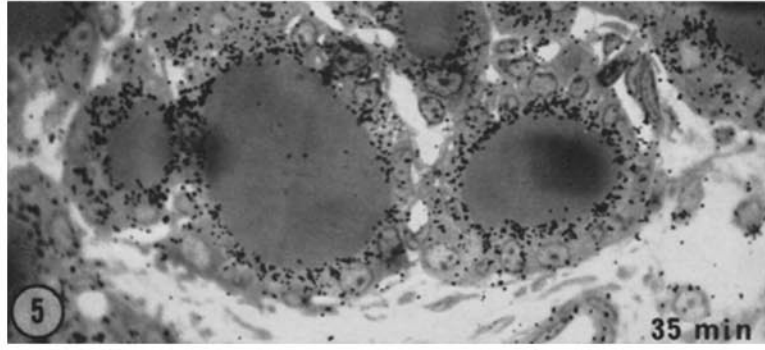
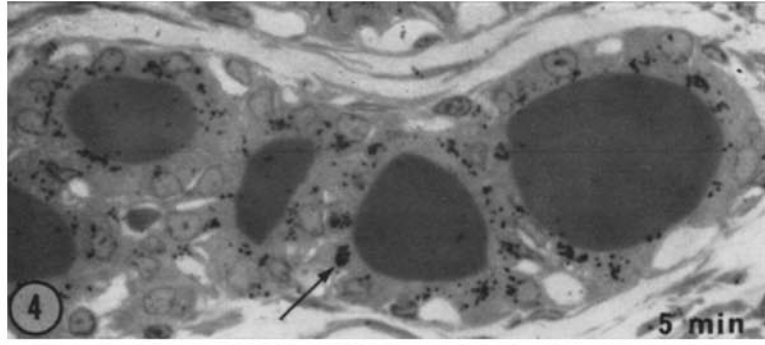
FIGURE 4 At 5 min after injection of fucose-³H, the majority of silver grains are localized in small clumps in the apical cytoplasm of the follicular cells (arrow).

FIGURE 5 By 35 min, the grains are distributed fairly evenly over the apical cytoplasm.

FIGURE 6 By 1 hr, the silver grains accumulate along the apical surface.

FIGURE 7 By 4 hr the label concentrated along the apical surface encroaches over the colloid more than at 1 hr, whereas the reaction over follicular cells is weaker than at 1 hr.

FIGURE 8 By 30 hr the follicular cells are almost devoid of silver grains. There is some migration of label away from the cell-colloid interface towards the central region of the colloid.



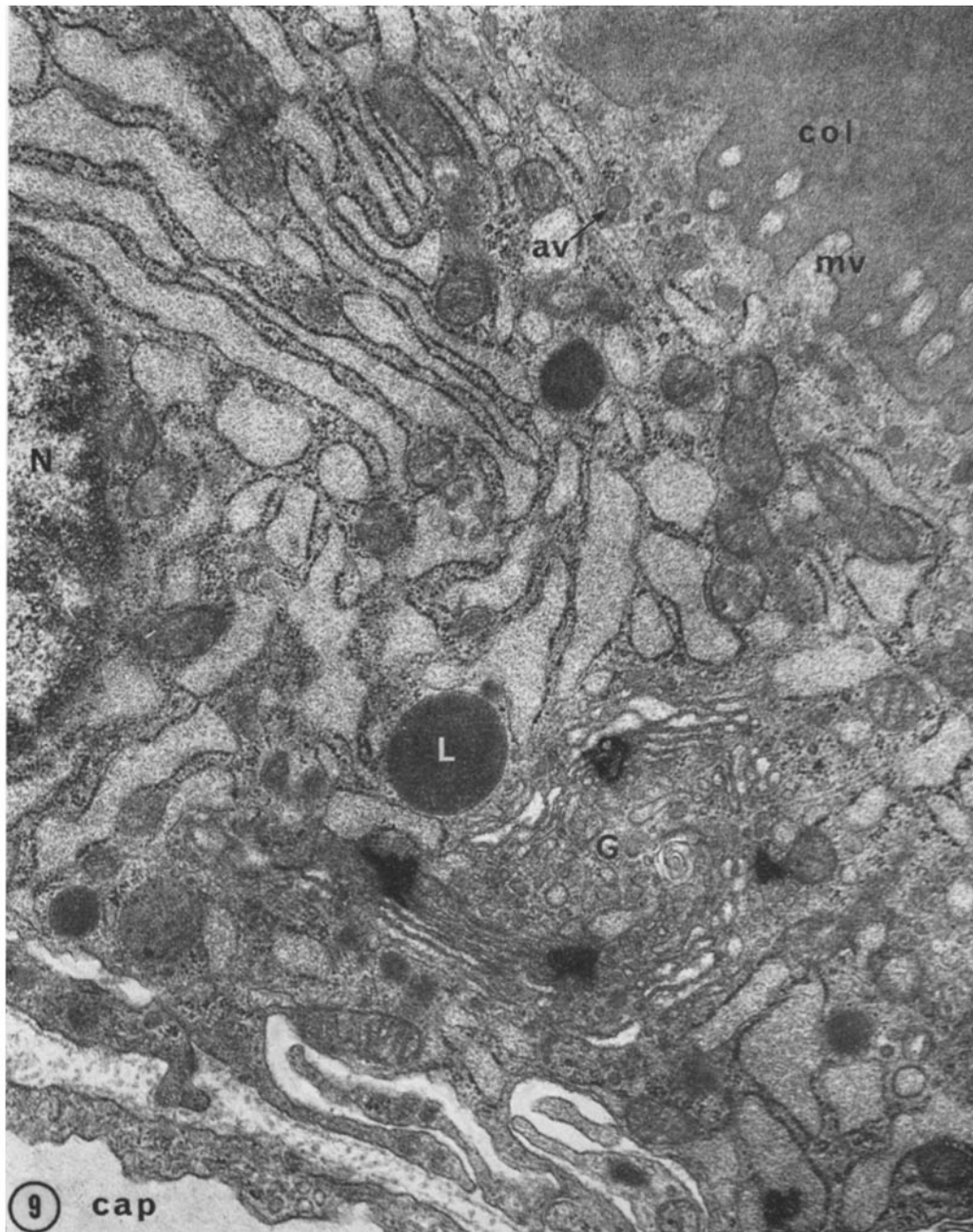


FIGURE 9 Electron microscope radioautograph of a thyroid follicular cell 5 min after injection of fucose- ^3H in vivo. This cell shows at left the nucleus (*N*), in lower center the Golgi apparatus (*G*), and a large dense body presumably lysosomal in nature (*L*), in the lower left corner a capillary (*cap*), and at upper right the apical cell border with a few microvilli (*mv*) below which a few apical vesicles may be distinguished (*av*). The colloid (*col*) is visible beyond the cell surface. Five silver grains are present in the picture, four of them overlying the Golgi apparatus, and one overlying the rough endoplasmic reticulum (lower right corner). Of the four Golgi grains, three are over the stacks of saccules while the one at the right seems to be over intermediate vesicles. The central region of the Golgi apparatus, which contains mostly condensing vacuoles, is not labeled. $\times 23,000$.

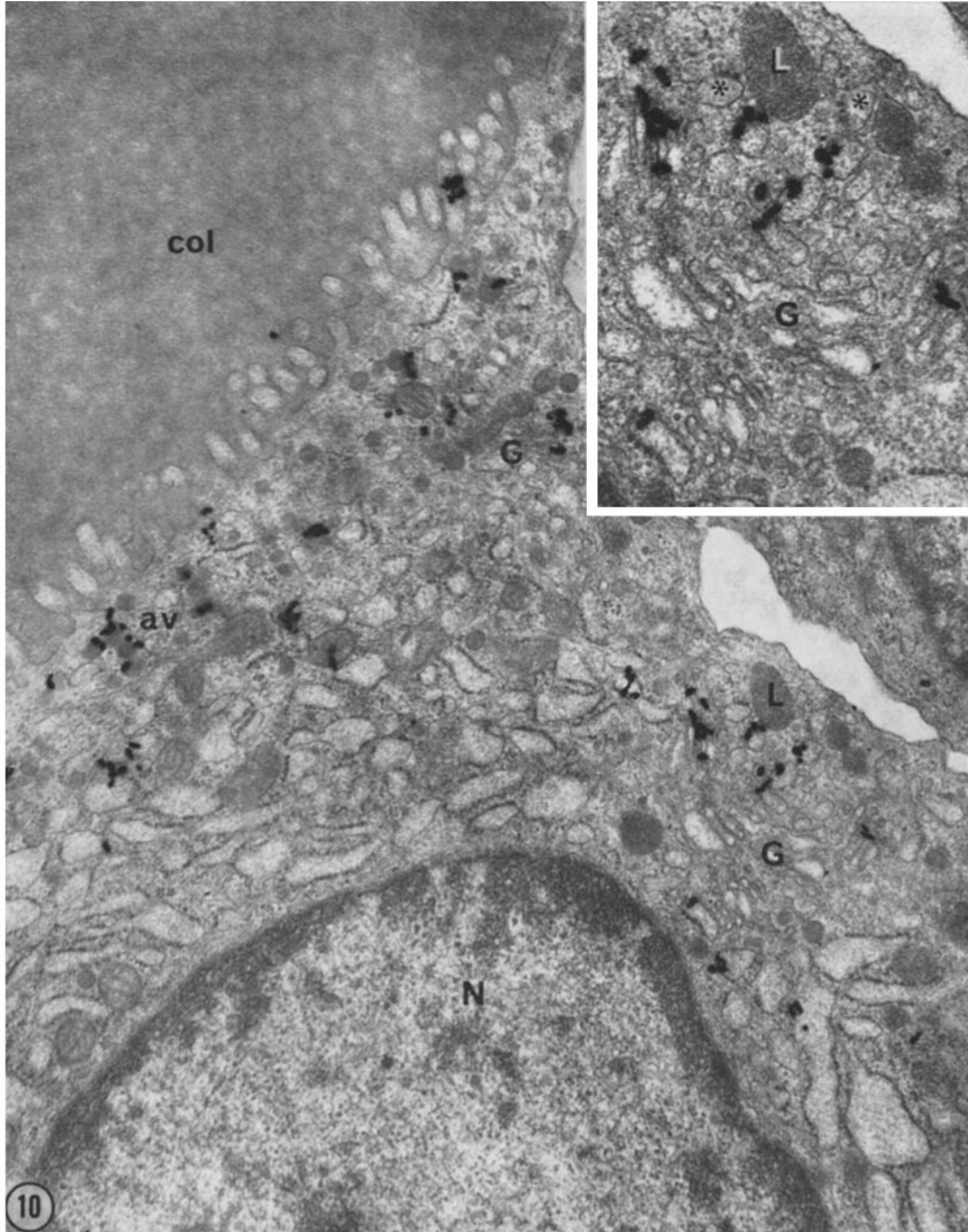
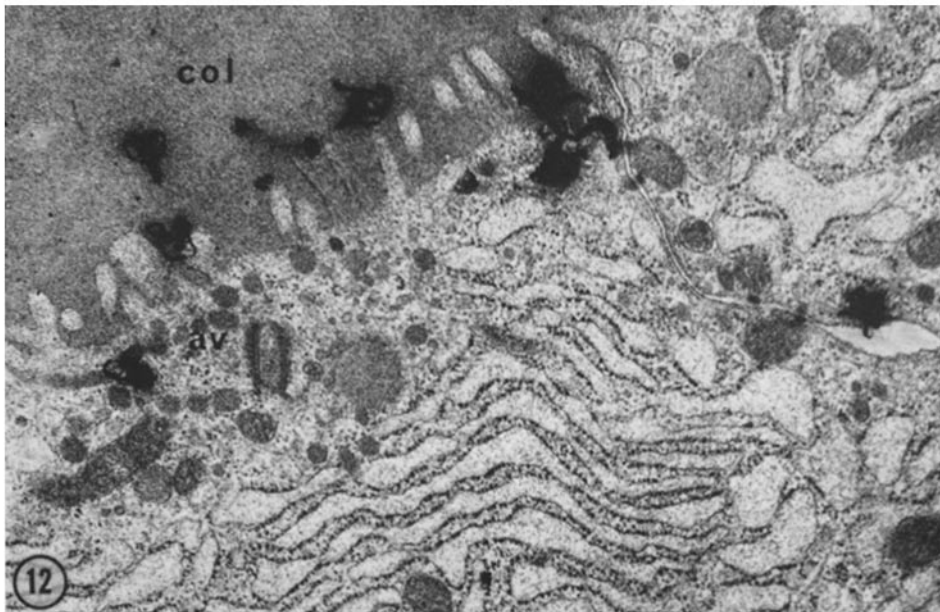
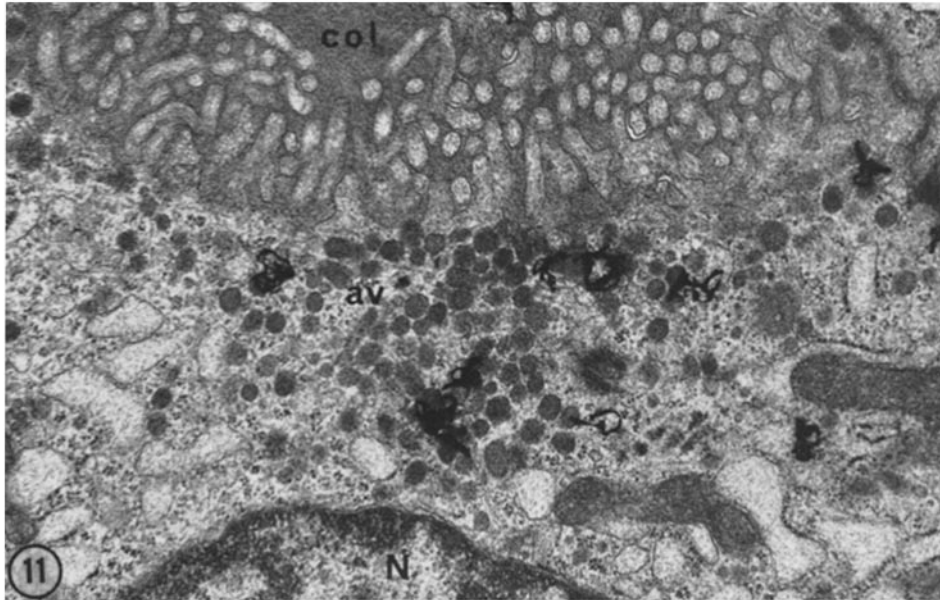


FIGURE 10 Electron microscope radioautograph of a thyroid follicular cell 35 min after injection of fucose-³H. This figure, unlike the others, is taken from a radioautograph developed in Elon-ascorbic acid, a method which yields small but often fragmented silver grains. Silver grains are present over the two Golgi stacks (G). The stack at lower right center is enlarged in the *insert* (× 40,000). It consists of saccules arranged in a semi-circle with the concavity oriented upwards. Between the saccules and a group of dense bodies (L), there are numerous condensing vacuoles, several of which are overlaid with silver grains (not to be confused with two rER cisternae indicated by asterisks). Most of the silver grains outside the Golgi region are over or near apical vesicles (av). Two grains are over the microvilli-colloid region. Some of the grains in the lower right corner may be related to the rER. N, nucleus; col, colloid; L, lysosome. × 23,000.



FIGURES 11-12 Electron microscope radioautographs of thyroid follicular cells 1 hr after injection of fucose-³H. $\times 23,000$.

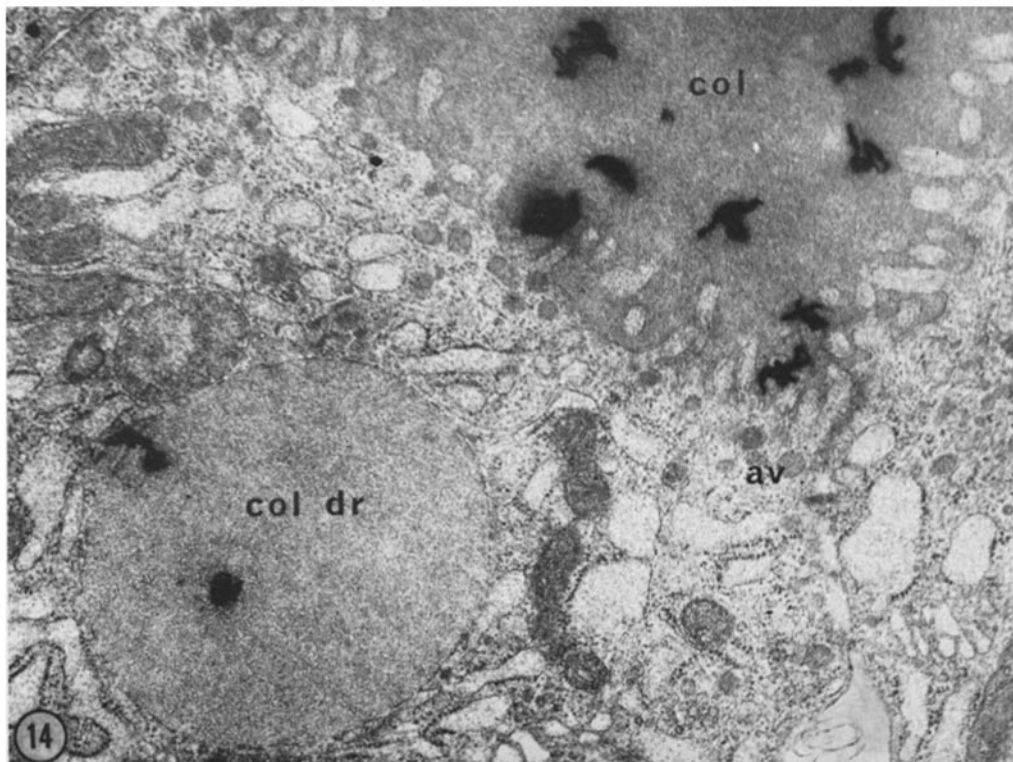
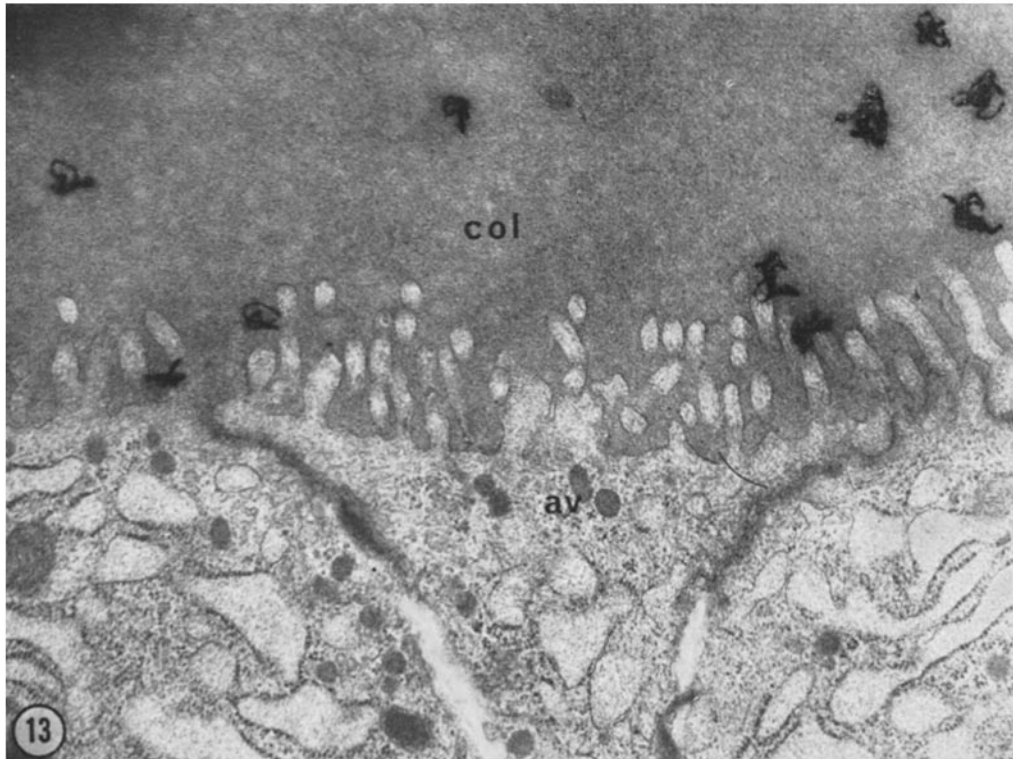
FIGURE 11 The majority of silver grains are localized over the numerous apical vesicles (*av*) which appear in this follicular cell. At top, one grain is over the colloid (*col*) in a region showing many microvilli. *N*, nucleus.

FIGURE 12 This figure shows a cell in which a few silver grains lie over apical vesicles (*av*), many grains are present also over the nearby colloid (*col*). (A centriole is at the right of the letters *av*.)

FIGURES 13-14 Electron microscope radioautographs of thyroid follicular cells 4 hr after injection of fucose-³H. *av*, apical vesicles. $\times 23,000$.

FIGURE 13 The three follicular cells whose apices appear in this figure are devoid of label, but silver grains abound over the colloid (*col*).

FIGURE 14 The majority of silver grains are concentrated over the colloid (*col*). In addition, two silver grains are seen over a colloid droplet (*col dr*). This observation confirms that the colloid in these droplets comes from resorbed luminal colloid.



of the rough endoplasmic reticulum. Condensing vacuoles and apical vesicles were unreactive (Fig. 9).

The concentration of silver grains over the Golgi apparatus was still prominent at 20 min, but by this time silver grains had appeared over condensing vacuoles and apical vesicles within the Golgi region. Some of the apical vesicles of the cell apex were also labeled. At 35 min, the concentration of grains over the Golgi apparatus decreased, while that over condensing vacuoles and apical vesicles increased (Fig. 10). At 1 hr, silver grains abounded over the apical vesicles (Fig. 11), as well as over the colloid close to the cell apex (Fig. 12). By 4 hr, nearly all silver grains were over the colloid (Fig. 13); the few grains that remained inside the cell were mainly over apical vesicles, but occasionally they were seen over colloid droplets (Fig. 14). By 30 hr, practically all grains were over the colloid or, when in cells, over colloid droplets.

QUANTITATIVE ANALYSIS OF RADIOAUTOGRAPHS: The grain counts carried out in the *light microscope* on semi-thin Epon sections (Table I) showed an increase between 3–5 and 20 min. The counts at 20, 35 min, and 1 hr were close to each other while those at 4 hr were somewhat higher. However, the difference between the 4 hr values and the others was not necessarily significant since there was only one animal per group. (The standard errors refer only to local variations in counts.)

At all times after injection of fucose-³H, examination of light microscope and electron microscope radioautographs revealed that practically all of

the grains were either over the supranuclear region of the follicular cells or over the colloid very close to the cell apex, there being very few grains over the base of the cells. This observation justifies the use of only the supranuclear region of the cells and the periphery of the colloid for quantitation in the electron microscope.

Examination of the “exclusive” silver grains in the crude counts (Table II) revealed that silver grains predominated over the Golgi apparatus at 3–5 min after injection but became less and less numerous over this organelle as time went on, whereas they increased over apical vesicles. The colloid showed a considerable increase in grain count at 1 hr and especially at 4 hr. (The “shared” grains were considered only for the corrected counts to be presented below.)

In Vitro Experiments

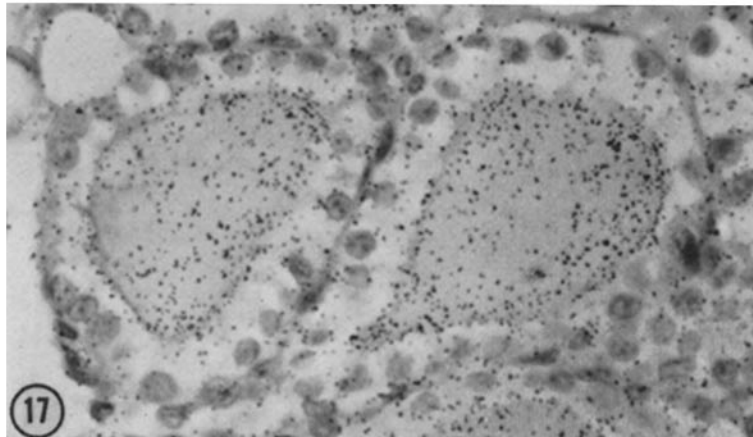
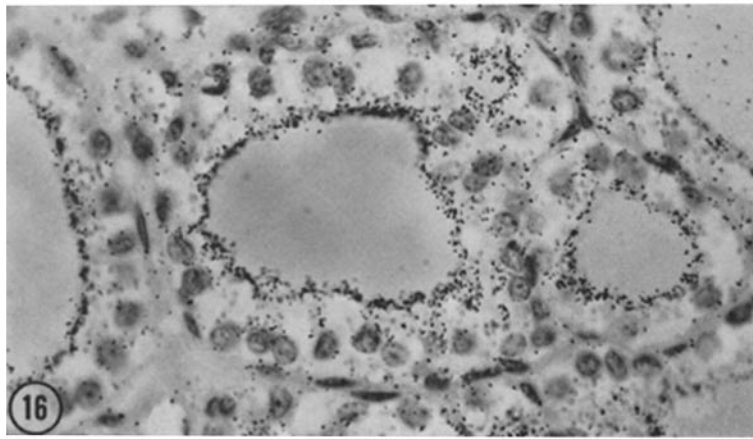
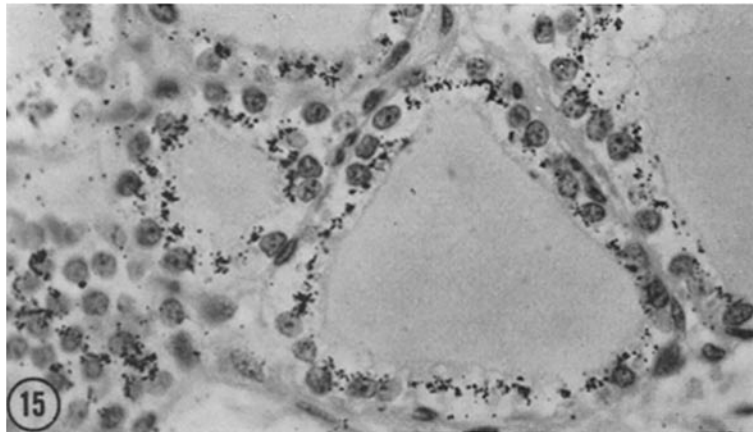
LIGHT MICROSCOPE RADIOAUTOGRAPHY: The thyroid lobes incubated for 5 and 15 min in medium containing radioactive fucose and fixed immediately showed a radioautographic reaction which was stronger and more uniform at 15 than at 5 min, and was observed in peripheral and central follicles at both times, so that the label had diffused throughout the thyroid lobe. The silver grains were concentrated in small regions beside the nucleus of follicular cells (Fig. 15). By 45 min (15 min pulse in a labeled medium followed by 30 min chase) the silver grains were mainly over the cell (Fig. 16), although a few were seen over the colloid in some follicles. The apex and the colloid reactions increased with time. After an

FIGURES 15–17 Light microscope radioautographs of rat thyroid lobes pulse-labeled *in vitro* for 15 min in medium containing fucose-³H, followed by incubation for various time intervals in chase medium containing unlabeled fucose. The recorded time is the total incubation time, that is, the 15 min pulse plus the length of the chase incubation. The preparations are stained with hematoxylin and eosin following radioautography. Each figure shows several follicles, with the central colloid area surrounded by follicular cells $\times 625$.

FIGURE 15 A portion of a thyroid lobe fixed at the end of the 15 min pulse-labeling in fucose-³H medium. The majority of silver grains are localized in small clumps over the apical region of follicular cells.

FIGURE 16 A portion of a thyroid lobe incubated for a total of 45 min. The silver grains accumulate along the apical surfaces of follicular cells.

FIGURE 17 A portion of thyroid lobe incubated for a total of 4 hr and 15 min. The follicular cells are almost devoid of grains. The label has migrated out of the cells and appears evenly dispersed throughout the colloid.



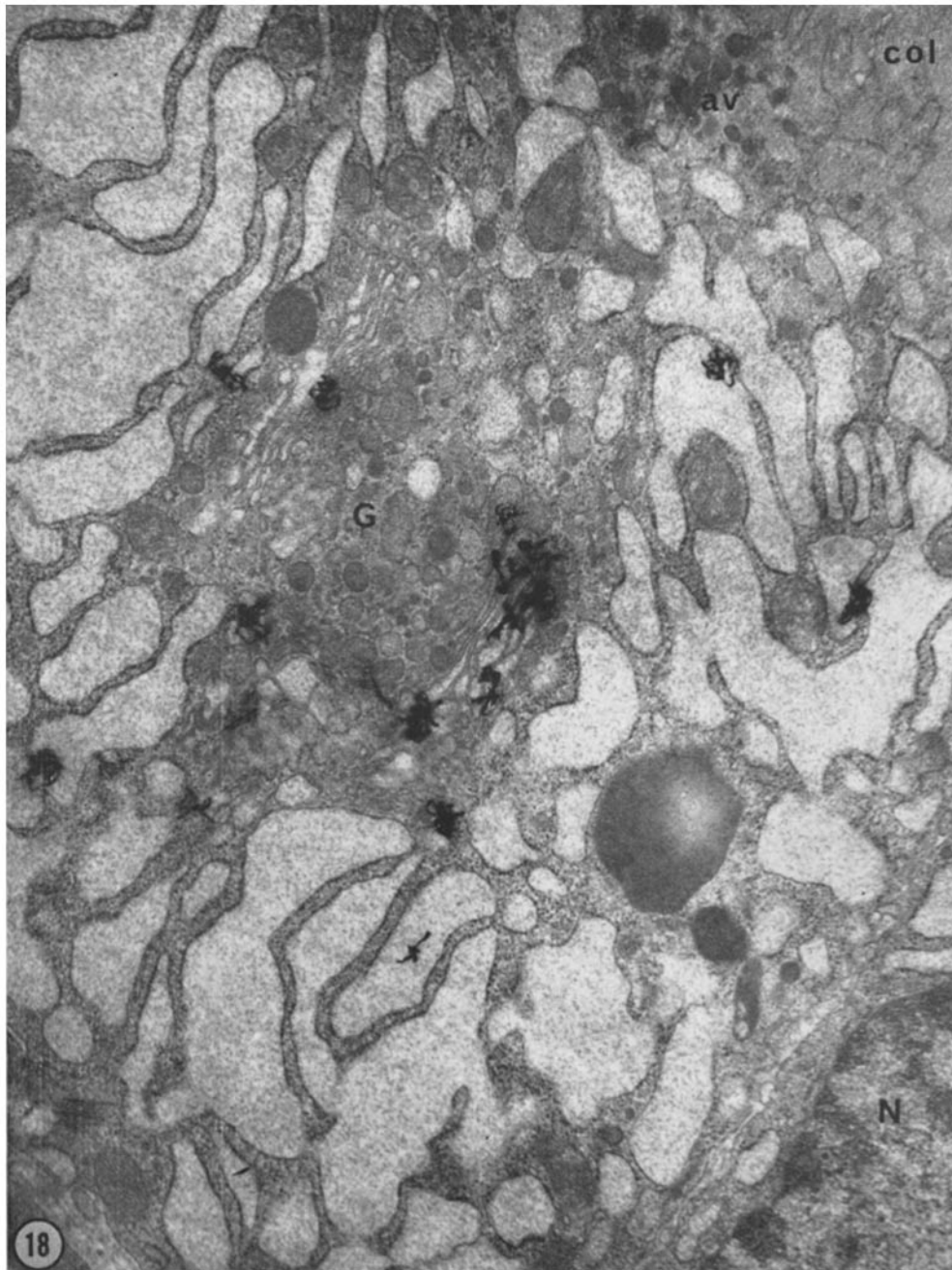
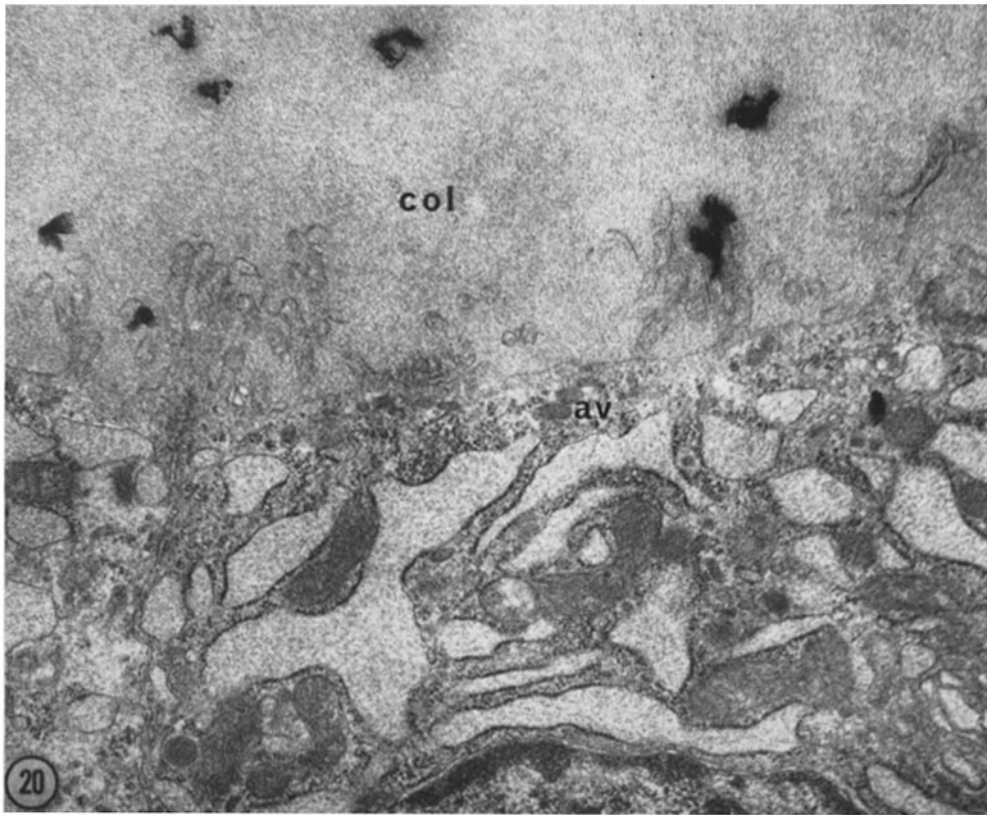
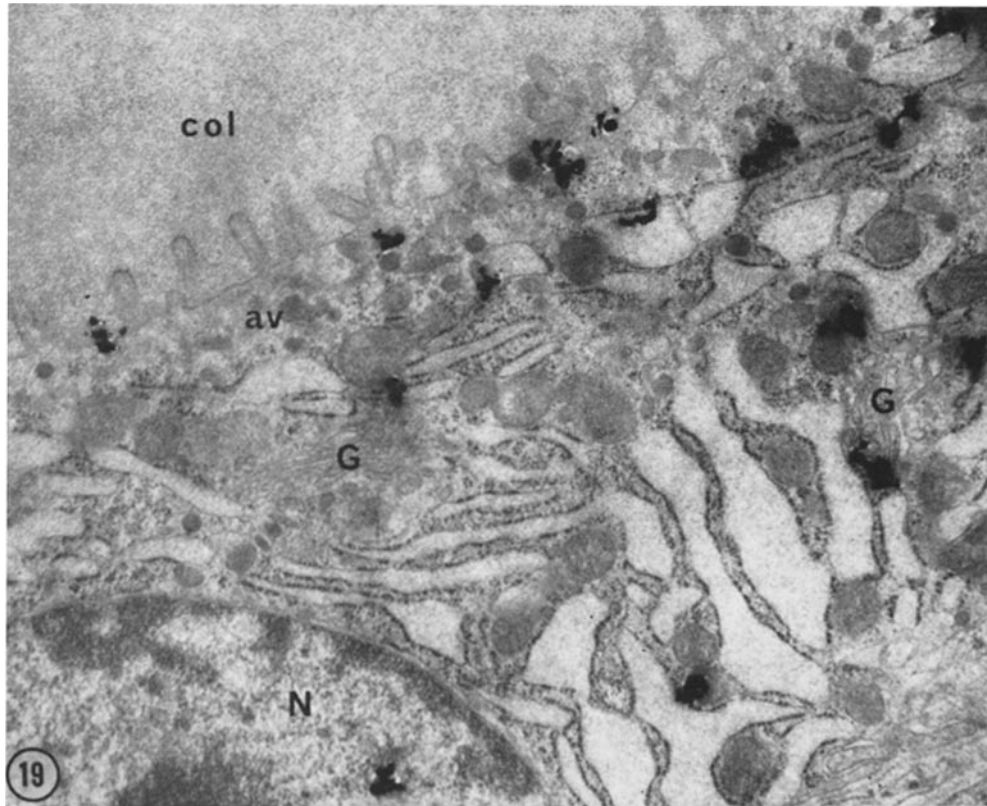


FIGURE 18 Electron microscope radioautograph of a thyroid follicular cell from a rat thyroid lobe fixed at the end of a 15 min pulse-labeling in vitro in a medium containing fucose-³H. Next to the colloid at upper right (*col*), a group of apical vesicles (*av*) shows no label. The majority of silver grains are over a large circular Golgi apparatus (*G*), predominantly over the stacks of saccules. Of the group of condensing vacuoles and apical vesicles occupying the center of the Golgi apparatus, only those next to the saccules are labeled. *N*, nucleus. $\times 23,000$.

FIGURES 19–20 Electron microscope radioautographs of thyroid follicular cells from rat thyroid lobes pulse-labeled for 15 min in medium containing fucose-³H and subsequently incubated for different periods in chase medium containing unlabeled fucose. The total time of incubation, including the 15 min pulse and the duration of the chase incubation, is recorded. *col*, colloid; *av*, apical vesicles; *N*, nucleus. $\times 23,000$.

FIGURE 19 Follicular cell incubated for a total of 30 min to show the migration of label. Although a few grains are still localized over the two Golgi stacks (*G*), the majority of them is in the apical region of the cell and may be related to apical vesicles.

FIGURE 20 Follicular cell incubated for a total of 1 hr and 15 min. By this time much of the label has migrated to the colloid.



interval of 2 hr and 15 min, only the colloid close to the cell apex was overlaid with silver grains. The colloid became uniformly labeled by 4-6 hr of incubation (Fig. 17). Only after such long periods of incubation did necrosis appear in the center of the thyroid lobe, in which case observations were limited to the intact rows of follicles at the periphery of the lobe.

ELECTRON MICROSCOPE RADIOAUTOGRAHY: At 5 and 15 min after the onset of incubation with fucose-³H, silver grains representing the fucose label were localized mainly over the Golgi apparatus of thyroid follicular cells, a few grains appearing also over the rough endoplasmic reticulum (Fig. 18). By 30 min (15 min pulse and 15 min chase) the number of silver grains decreased over the Golgi apparatus but increased over apical vesicles (Fig. 19). While a few grains were observed at this time over the colloid adjacent to microvilli, many more appeared at later times (Fig. 20). By 4 and 6 hr, the grains were found almost exclusively over the colloid.

QUANTITATIVE ANALYSIS: The crude counts of "exclusive" grains in Table IV showed a

Golgi reaction initially, with a fair number of grains appearing over apical vesicles at 30 min (15 min pulse, 15 min chase) and over the colloid by 135 min.

DISCUSSION

Both the *in vivo* and the *in vitro* series of experiments demonstrated that fucose-³H was initially taken up into the thyroid follicular cells (Figs. 4, 15), and that later the label appeared in the colloid (Figs. 8, 17). As pointed out in the Introduction, fucose injected into the rat is incorporated as such into glycoproteins with no significant conversion into other substances (Coffey et al., 1964; Bekesi and Winzler, 1967; Kaufman and Ginsburg, 1968). It is also known that some of the carbohydrate side chains of thyroglobulin terminate with fucose residues (Spiro and Spiro, 1965; Spiro, 1965) and that within 5 or 10 min after incubation of rat thyroids with fucose-³H most of the label is incorporated as fucose residues into a 17-18S protein which corresponds to non-iodinated thyroglobulin (Herscovics, 1970). Since

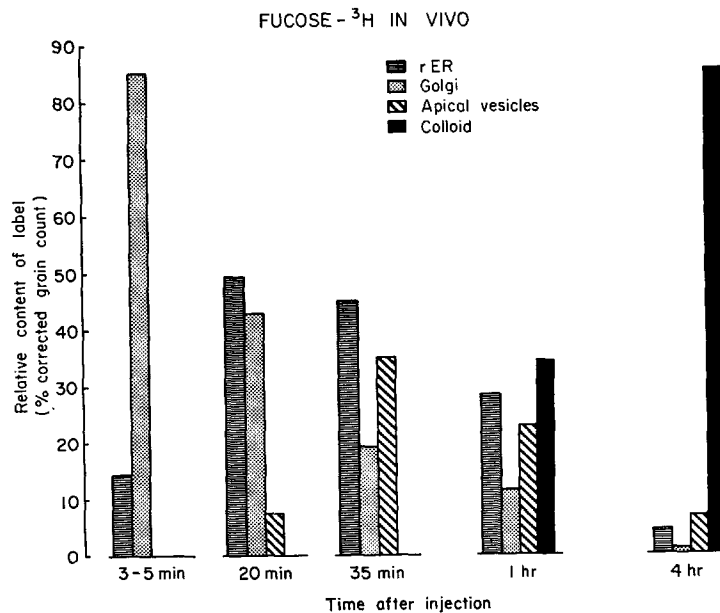


FIGURE 21 The grain counts over various cell structures and over colloid were classified as either exclusive or shared by two or more of these structures. The shared counts were analyzed and attributed on the basis of probability to only one structure. The corrected grains were the sum of exclusive and adjusted shared grains. The relative number of grains per rough endoplasmic reticulum (*rER*), Golgi apparatus, apical vesicles, and colloid were then compared as a percentage of the total number of corrected grains over these four structures at the various times after injection of fucose-³H.

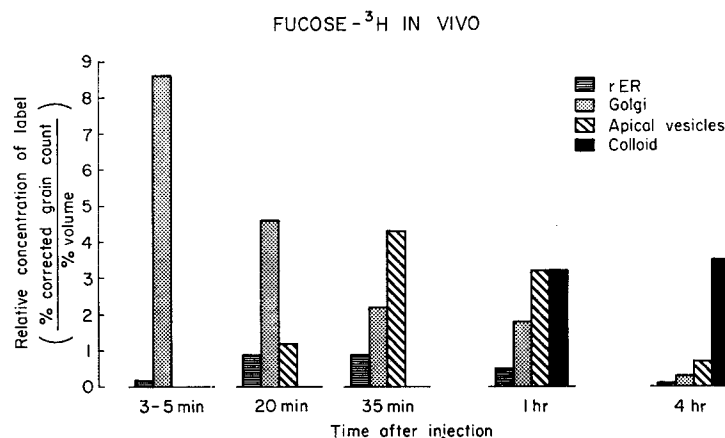


FIGURE 22 The relative percentage of corrected grains per *rER*, Golgi apparatus, apical vesicles, and colloid (see Fig. 21) was divided respectively by the relative volume of each structure as determined histometrically to yield the relative concentration of label in each structure at the various times after injection of fucose-³H. Relative concentration figures emphasize the importance of the role of Golgi apparatus, apical vesicles, and colloid, but minimize that of the rough ER.

radioautographs prepared at such early times showed that the label was restricted to the cell (Figs. 4, 18), it may be concluded that fucose was incorporated into thyroglobulin inside the thyroid follicular cells. Furthermore, at later times, labeled thyroglobulin was released into the follicular lumens, in keeping with the finding that thyroglobulin makes up the bulk of the colloid (Smeds, 1970).

Intracellular Site of Fucose Incorporation

At 3 and 5 min after injection, corrected counts revealed that as much as 85% of the silver grains was present over the Golgi apparatus (Fig. 21). Furthermore, calculation of the relative concentration of label proved that the Golgi apparatus was the only cell organelle to contain a significant amount at these times (Fig. 22). Thus, the Golgi apparatus must be the main site of incorporation of fucose. Since this sugar is terminal, it follows that some of the carbohydrate side chains of thyroglobulin are completed in the Golgi apparatus.² According to current concepts of glycoprotein

² After injection of the labeled sugars, galactose and fucose (and in some cases, glucose), radioautography showed that the label was incorporated into the Golgi apparatus of many cells other than thyroid follicular cells (Peterson and Leblond, 1964; Droz, 1966; Neutra and Leblond, 1966 *a*, 1966 *b*; Bennett, 1967; Weinstock, 1969; Bennett, 1970; Bennett and Leblond, 1970; Zagury et al., 1970).

biosynthesis (Gottschalk, 1969; Spiro, 1969), the site of incorporation of a sugar should harbor the endogenous acceptor, the nucleotide sugar, and the effective enzyme, that is, respectively in this case, fucose-free thyroglobulin, guanidine diphosphate (GDP)-fucose, and fucosyltransferase. Recently, in HeLa cells, fucosyltransferase activity has been localized to smooth internal membranes, which probably contain the Golgi apparatus (Bosmann et al., 1968). Within the Golgi apparatus, the site of the incorporation of fucose into thyroglobulin seems to be the saccule, since almost invariably at the 3- and 5-min time intervals the silver grains were over the stacks of saccules (Fig. 9).

Despite the evidence for the Golgi apparatus as the site of incorporation of fucose, consideration has to be given to the grains present in the rough ER. Analysis of the crude grain counts *in vivo* (Table II) revealed that 83-91% of the grains assigned to the rough ER were "shared" with apical vesicles or Golgi apparatus, especially at the times when these organelles were themselves labeled. Of the "exclusive" grains over the rough ER at the 3-5 min interval, 20 out of 29 were within 5000 Å from the Golgi apparatus. At the 15 min time interval *in vitro*, most of the exclusive grains over the rough ER were also fairly close to the Golgi apparatus (Fig. 18). These grains may be interpreted as being due to incorporation of fucose-³H into unfinished molecules of thyroglobulin just

before they enter the Golgi apparatus; but the uptake of fucose in this location is very small.

Migration of the Label

Fucose disappears from the blood, since by 20 min after intravenous injection the level of fucose label in the plasma is low (Bocci and Winzler, 1969) and the total amount of radioactivity inside the tissues does not increase after this time (Coffey et al., 1964). Counts in the light microscope (Table I) indicated no increase in the thyroid gland after the 20 min interval, so that any change in the distribution of label within the follicle after that time must be the result of transfer of label from one site to another.

While at 3 and 5 min the Golgi reaction consisted almost exclusively of silver grains overlying the stacks of saccules, by 20 and 35 min it extended to grains over nearby condensing vacuoles and apical vesicles (Fig. 10). In particular, the concentration of label in apical vesicles increased nearly four times between 20 and 35 min, while at the same time the concentration in the Golgi apparatus decreased markedly (Fig. 23). The increasing radioactivity of apical vesicles must, therefore, be due to the migration of pre-labeled thyroglobulin from Golgi apparatus to apical vesicles, and not to direct fucose incorporation by these vesicles. Presumably, condensing vacuoles arose on the mature face of the Golgi apparatus from the innermost saccules and, in so doing,

received the labeled thyroglobulin contained in those saccules. Soon after, condensing vacuoles transformed into apical vesicles. After the 35 min interval the concentration of label in apical vesicles decreased, while that in the colloid increased. In the *in vitro* series similar results were obtained (Fig. 24), except that the radioactivity diffused through the colloid faster than it did *in vivo* (perhaps because of the age difference of the animals used).

Since the fucose-labeled thyroglobulin was transferred to the colloid by way of the apical vesicles (Fig. 11), these functioned as secretion granules, as suggested previously (Nadler et al., 1964; Whur et al., 1969). While the mechanism by which their content is released into the follicle lumen is not clear, it is likely to be by a process of exocytosis. When examined under high power after glutaraldehyde fixation (Fig. 2), the content of the apical vesicles displayed the same pattern as the colloid. This observation confirmed that the apical vesicles are the source of the colloid.

Sequence of Events in the Elaboration of Thyroglobulin

In the rat thyroid follicular cells, the polypeptide components of thyroglobulin are formed in relation with ribosomes, pass into the cisternae of the rough ER, migrate from there to the Golgi apparatus, and eventually are secreted into the

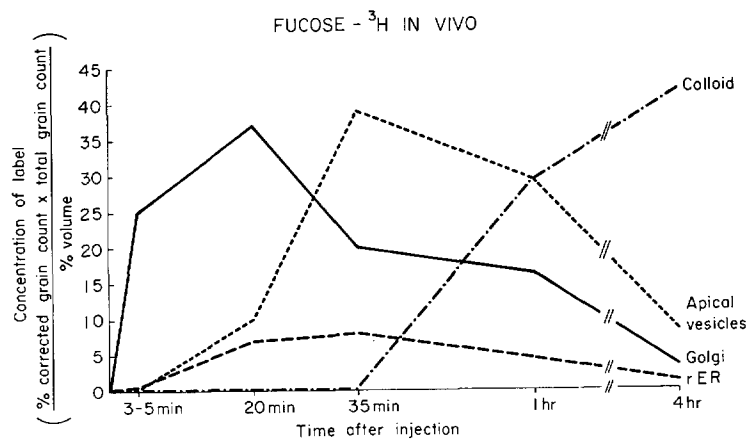


FIGURE 23 The relative concentration of label in *rER*, Golgi apparatus, apical vesicles, or colloid (see Fig. 22) at each time interval after injection of fucose-³H was multiplied by the total grain counts over cells and colloid (see Table I) to yield the concentration of label. This method indicates how the concentration of label in the organelles varies with time after fucose-³H injection.

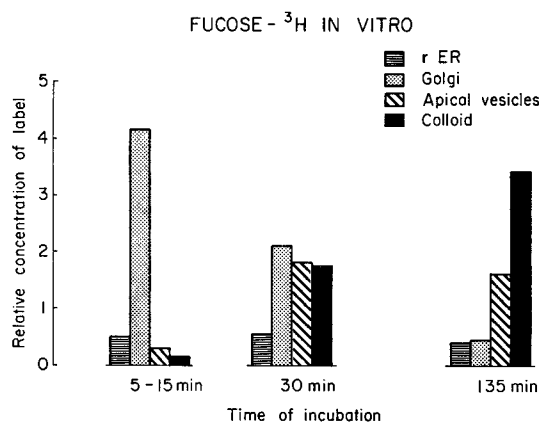


FIGURE 24 The relative percentage of corrected grains in rER, Golgi apparatus, apical vesicles, and colloid at each time interval after incubation in fucose-³H was divided by the relative volume of each structure to yield the relative concentration of label. (The time of incubation includes 15 min in medium containing fucose-³H, followed by different periods of time in chase medium.) The label decreases in the Golgi apparatus with time, while increasing at first in apical vesicles and later in the colloid.

colloid (Nadler et al., 1964). The relation of these events to the building up of the carbohydrate side chains will be briefly reviewed.

(a) Within the *rough ER*, the polypeptide components of thyroglobulin acquire mannose (Whur et al., 1969) and glucosamine,³ in agreement with the finding of mannosyl- and *N*-acetylglucosaminyltransferase activities in the rough microsomes of sheep thyroid (Bouchilloux et al., 1970). The incorporation of these two sugars probably takes place after passage into the cisternae (Bouchilloux and Cheftel, 1966). Of the two types of carbohydrate side chains, the smaller one, which consists of mannose and *N*-acetylglucosamine, is probably completed within the rough ER, while the larger side chain is not. In the rough ER (and perhaps in the intermediate vesicles which are believed to carry incomplete thyroglobulin into the outermost Golgi saccules), there may be a minor incorporation of galactose (Haddad, 1971). According to the present results, the incorporation of fucose is so small that its significance is doubtful.

³ Haddad, A., M. D. Smith, A. Herscovics, and C. P. Leblond. 1971. Radioautographic study of the incorporation of ³H-glucosamine by rat thyroids *in vitro* in relation to the elaboration of thyroglobulin. Manuscript in preparation.

(b) within the *saccules of the Golgi apparatus*, there is incorporation of most of the galactose (Whur et al., 1969; Haddad, 1971) and, as shown by the present investigation, of fucose. Galactosyltransferase (but, so far, not fucosyltransferase) activity has been detected in the Golgi-rich fraction of thyroid cells (Bouchilloux et al., 1969, 1970). The addition of fucose within the Golgi apparatus indicates completion of the side chains ending with this sugar. It is possible, although evidence is lacking, that sialic acid-terminated side chains are also completed there.

(c) *Condensing vacuoles* containing the completed glycoprotein arise from the innermost (mature) saccule of Golgi stacks and, soon after, are transformed into *apical vesicles*. These apical vesicles function as secretion granules, since they transport the glycoprotein towards the *follicular lumen* where it is deposited as colloid.⁴

The assistance of Dr. Beatrix Kopriwa with the radioautographic work is acknowledged.

The authors wish to acknowledge the support of the Medical Research Council of Canada and of Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil.

Received for publication 14 August 1970, and in revised form 30 October 1970.

REFERENCES

- ARIMA, T., M. J. SPIRO, and R. G. SPIRO. 1970. Structural investigations on the carbohydrate units of human and calf thyroglobulins. *Fed. Proc.* **29**:600.
- BASERGA, R., and D. MALAMUD. 1969. Autoradiography. Techniques and Application. Harper & Row, Publishers, New York. 254.
- BEKESI, J. G., and R. J. WINZLER. 1967. The metabolism of plasma glycoproteins. Studies on the in-

⁴ After thyroglobulin reaches the lumen, it is iodinated, resulting in the formation of thyroxyl and triiodothyronyl residues. The release of free thyroxine and triiodothyronine from thyroglobulin seems to occur after the uptake of small portions of colloid into the cell in the form of colloid droplets (Nadler et al., 1962). The present results reveal that the colloid droplets observed at early times (3-5, 20, and 35 min) were not labeled, whereas those observed at 4 and 30 hr were labeled as shown in Fig. 14. Thus, the colloid droplets were labeled only when the colloid was labeled, an observation confirming the early findings of Nadler et al. (1962).

- corporation of L-fucose-1-¹⁴C into tissue and serum in the normal rat. *J. Biol. Chem.* **242**:3873.
- BENNETT, G. C. 1967. A radioautographic study of the distribution of labelled sugars in rat tissues. M. Sc. Thesis, Department of Anatomy, McGill University.
- BENNETT, G. 1970. Migration of glycoprotein from Golgi apparatus to cell coat in the columnar cells of the duodenal epithelium. *J. Cell Biol.* **45**:668.
- BENNETT, G., and C. P. LEBLOND. 1970. Formation of cell coat material for the whole surface of columnar cells in the rat small intestine, as visualized by radioautography using L-fucose-³H. *J. Cell Biol.* **46**:409.
- BOCCI, V., and R. J. WINZLER. 1969. Metabolism of L-fucose-1-¹⁴C and of fucose glycoproteins in the rat. *Amer. J. Physiol.* **216**:1337.
- BOSMANN, H. B., A. HAGOPIAN, and E. H. EYLAR. 1968. Glycoprotein biosynthesis: the characterization of two glycoproteins: fucosyl transferases in HeLa cells. *Arch. Biochem. Biophys.* **128**:470.
- BOUCHILLOUX, S., O. CHABAUD, M. MICHEL-BECHET, M. FERRAND, and A. M. ATHOUËL-HAON. 1970. Differential localization in thyroid microsomal subfractions of a mannosyltransferase, two N-acetylglucosaminyl transferases and a galactosyltransferase. *Biochem. Biophys. Res. Com.* **40**:314.
- BOUCHILLOUX, S., and C. CHEFTEL. 1966. Biosynthesis of thyroglobulin: absence of ¹⁴C-glucosamine incorporation on thyroid polysomes. *Biochem. Biophys. Res. Commun.* **23**:305.
- BOUCHILLOUX, S., M. FERRAND, J. GREGOIRE, and O. CHABAUD. 1969. Localization in smooth microsomes from sheep thyroid of both a galactosyltransferase and an N-acetylhexosaminyltransferase. *Biochem. Biophys. Res. Comm.* **37**:538.
- COFFEY, J. W., O. N. MILLER, and O. Z. SELLINGER. 1964. The metabolism of L-fucose in the rat. *J. Biol. Chem.* **239**:4011.
- DROZ, B. 1966. Elaboration de glycoprotéines dans l'appareil de Golgi des cellules hépatiques chez le rat; étude radioautographique en microscopie électronique après injection de galactose-³H. *C. R. Acad. Sci. Ser. D.* **262**:1766.
- GOTTSCHALK, A. 1969. Biosynthesis of glycoproteins and its relationship to heterogeneity. *Nature (London)*. **222**:452.
- HADDAD, A. 1971. In vivo incorporation of ³H-galactose by thyroid follicular cells of the rat. *Anat. Rec.* In press.
- HERSCOVICS, A. 1969. Biosynthesis of thyroglobulin. Incorporation of (1-¹⁴C)-galactose, (1-¹⁴C)-mannose and (4,5-³H)-leucine into soluble proteins by rat thyroids *in vitro*. *Biochem. J.* **112**:709.
- HERSCOVICS, A. 1970. Biosynthesis of thyroglobulin: incorporation of (³H) fucose into proteins by rat thyroids *in vitro*. *Biochem. J.* **117**:411.
- JENSEN, F. C., R. B. L. GWATKIN, and J. D. BIGGERS. 1964. A simple organ culture method which allows simultaneous isolation of specific types of cells. *Exp. Cell Res.* **34**:440.
- KAUFMAN, R. L., and V. GINSBURG. 1968. The metabolism of L-fucose by HeLa cells. *Exp. Cell Res.* **50**:127.
- LUPULESCU, A., and A. PETROVICI. 1968. Ultrastructure of the Thyroid Gland. S. Karger AG., Basel, Switzerland.
- NADLER, N. J., S. K. SARKAR, and C. P. LEBLOND. 1962. Origin of intracellular colloid droplets in the rat thyroid. *Endocrinology*. **71**:120.
- NADLER, N. J., B. A. YOUNG, C. P. LEBLOND, and B. MITMAKER. 1964. Elaboration of thyroglobulin in the thyroid follicle. *Endocrinology*. **74**:333.
- NEUTRA, M., and C. P. LEBLOND. 1966 *a*. Synthesis of the carbohydrate of mucus in the Golgi complex as shown by electron microscope radioautography of goblet cells from rats injected with glucose-³H. *J. Cell Biol.* **30**:119.
- NEUTRA, M., and C. P. LEBLOND. 1966 *b*. Radioautographic comparison of the uptake of galactose-³H and glucose-³H in the Golgi region of various cells secreting glycoproteins or mucopolysaccharides. *J. Cell Biol.* **30**:137.
- PETERSON, M., and C. P. LEBLOND. 1964. Synthesis of complex carbohydrates in the Golgi region, as shown by radioautography after injection of labeled glucose. *J. Cell Biol.* **21**:143.
- SMEDS, S. 1970. Protein composition of the colloid collected from single rat thyroid follicles. *Biochem. Biophys. Res. Comm.* **38**:1168.
- SPIRO, R. G. 1965. The carbohydrate units of thyroglobulin. *J. Biol. Chem.* **240**:1603.
- SPIRO, R. G. 1969. Glycoproteins: their biochemistry, biology and role in human disease. *New Engl. J. Med.* **281**:991.
- SPIRO, R. G., and M. J. SPIRO. 1965. The carbohydrate composition of the thyroglobulins from several species. *J. Biol. Chem.* **240**:997.
- SPIRO, R. G., and M. J. SPIRO. 1966. Glycoprotein biosynthesis: studies on thyroglobulin. Characterization of a particulate precursor and radioisotope incorporation by thyroid slices and particle systems. *J. Biol. Chem.* **241**:1271.
- WEINSTOCK, A. 1969. Sites of synthesis and pathway of migration of glycoprotein in the ameloblast. Ph.D. Thesis, Department of Anatomy, McGill University.
- WHUR, P., A. HERSCOVICS, and C. P. LEBLOND. 1969. Radioautographic visualization of the incorporation of galactose-³H and mannose-³H by rat thyroids *in vitro* in relation to the stages of thyroglobulin synthesis. *J. Cell Biol.* **43**:289.
- WISSIG, S. L. 1960. The anatomy of secretion in the

follicular cells of the thyroid gland. I. The fine structure of the gland in the normal rat. *J. Biophys. Biochem. Cytol.* 7:419.

ZAGURY, D., J. W. UHR, J. D. JAMIESON, and G. E.

PALADE. 1970. Immunoglobulin synthesis and secretion. II. Radioautographic studies of sites of addition of carbohydrate moieties and intracellular transport. *J. Cell Biol.* 46:52.

APPENDIX

The Interpretation of Grain Counts in Electron Microscope

Radioautography

N. J. NADLER

INTRODUCTION

The silver grains present in electron microscope radioautographs are usually examined in photographs. The goal is to assign each grain to a structure visible in the photograph. Two problems are encountered. The first one is to find out the relation between the location of the grain and the source of the radioactivity which caused it. This can be achieved by various considerations (Caro, 1962; Granboulan, 1965; Miura and Mizuhara, 1965; Salpeter and Bachmann, 1965; Salpeter, et al., 1969; and another to be recorded in an article being prepared by the author, Nadler 1971). In general, by one theory or another, probabilities are calculated for the source being at different distances from the center of the grain, and a "resolution boundary" is defined as the distance beyond which the source of radioactivity is located at, say, less than 5% probability (or any other arbitrary designation). Once this distance is known, a circle may be drawn about the center of the silver grain in the photographic representation with this distance as radius; thus, the structure containing the radioactive label is expected to be within this circle with a probability of 95%.

Second, once this is accomplished, one may then find about some grains that there is only one structure visible within the "resolution boundary circle"—such grains are here being called "exclusive grains." On the other hand, about other grains, there is more than one structure visible within the circle—such grains are being called "shared grains." If all grains observed were exclusive, there would be little or no problem in interpretation; simply, the number of grains assigned to each class of structures would be pro-

portional statistically to the content of radioactive label in that class. However, as long as there are any shared grains, there will be a problem in interpretation (Williams, 1969). For, there is ample evidence that, on the average for ^3H , only one radioactive emission from the tissue can account for each developed silver grain in photographic emulsions normally used (Pelc, 1963). Thus, only one of the two or more biological structures related to a shared grain is in reality the source of radioactivity. In this article, a theory is presented as a basis for the analysis of shared grains, that is, to deduce what the probable distribution of radioactive label is among the classes of structures sharing grains. This has been applied to the set of experiments reported in this article (sample calculations are included for the 20 min data in vivo). The method is expected to be generally useful in the quantitative interpretation of electron microscope radioautographs.

THEORY AND METHOD

The selection of the photographic representation of electron microscope radioautographs ought to be in theory a random process. As long as a single silver grain is present, the investigator must not reject a photograph provided the structures adjacent to the grains can be identified, if necessary by inspection of the photographic negative in case of unsatisfactory printing. If the object is to determine the relative *content* of radioactive label in each class of structure where radioisotope is present, it is necessary to obtain electron microscope radioautographs illustrating the entire anatomical region where these classes exist; for

example, the classes might represent the organelles of a cell, so that entire cells would need to be scanned. If the aim is to determine the relative concentration of radioactive label in each of the classes, then, if it is correct to assume that the concentration in the structure of a given class is uniform throughout the anatomical region, it would be acceptable to restrict electron microscope radioautographs to localized areas so that the whole region need not be scanned.

Since the error in random counting is proportional to the square root of the number of counts, it is best, if feasible, to take into account not much less than 500 grains. Around each silver grain in the photographs selected, draw appropriate resolution boundary circles¹ to localize (with the designated probability) the site of origin of radioactivity with respect to each observed grain. Decide which class of structure (or classes of structures) is (or are) enclosed within each resolution boundary circle. Select for analysis of radioactive label content all classes of structures to which are assigned at least one exclusive silver grain. The rationale is that, if it is not possible to assign to a class of structure even one exclusive grain, it should also not be possible, because of the restrictions in resolving power, to infer with certainty that this class of structure necessarily contains radioactive label. If the number of shared grains associated with such structures is small, say, less than 5% of the total counted, then it may be decided simply to exclude this class from the analysis. If, on the other hand, the number of shared grains associated with such structures is not small, then other considerations ought to be applied to estimate the possibility that this class may yet contain radioactive label (Coimbra and Leblond, 1966; Williams, 1969). Moreover, if the number of exclusive grains assigned to a particular class is also small, say, less than 1% of the total, it may be decided likewise to ignore that class. If a grain is observed to be shared by a class of structure which is included in the analysis and by another class which is excluded, the grain is assigned only to the class included. Then, for each class, designated generally as n , rearrange the data to list:

(a) the sum of grain counts which are finally

¹ Nadler, N. J. 1971. Estimation of resolving power in electron microscope radiography. Manuscript in preparation.

assigned exclusively to that class, and designate as ${}_nE$; and

(b) the sum of grain counts which are finally assigned to that class but shared with one or more other classes; and designate every observed combination among n and any other class as ${}_n s_{n', n''}$, etc. where n', n'' , etc. denote whatever classes were found to be shared by n .

The object of the analysis is to attribute to each class of structure a true grain count ${}_nC$, where the total number of grains enumerated is

$$T_{EM} = \sum {}_nC.$$

It is obvious that, if a grain is shared, it will be counted more than once, so that the grand total of counts under (a) and (b) for all structures will exceed T_{EM} .

The problem to solve is what proportion of the grains shared by a combination of a number of classes can be attributed to each of the classes concerned. There are two factors which govern the probability that any silver grain which is shared by more than one class of structure would belong to a particular class and not the others.

The first factor is the true grain count for that class, ${}_nC$, compared with the others. Clearly, since a certain proportion of all grains is the result of radioactivity emitted from a given class of structure, this same proportion also reflects the probability that any given grain observed can be attributed to that structure. Therefore, according to this factor alone, the proportion of all grains shared, say, by classes n, n' , and n'' , which could be attributed to n alone would be

$$\frac{{}_nC}{{}_nC + {}_{n'}C + {}_{n''}C}.$$

The second factor has to do with the fact that the resolution boundary circles are not infinitesimal in size (diameter represented is of the order of 0.4 μ). Therefore, regardless of how much radioactive label they contain per unit volume, structures which happen to be spread out, like ribosomes and plasma membranes, might be included more often within resolution boundary circles than structures which are compact, like nuclei. To take account of the error introduced in assigning structures to shared grains as a result only of their diffuse shape, consider the following proposition. Measure the correct relative volume of the classes of structures under consideration by

using the "point hit" method (Chalkley, 1943) as follows. Employ a number (not less than 500) of random points (say, the intersections of a set of horizontal and vertical lines in photographs of the radioautographs); observe which structures are struck by the points, and count the number of "hits" for each class of structure. Express the number of "hits" as a percentage of the total number of points employed. Assuming uniform tissue section thickness, this is the relative true volume of each class, designated as ${}_nV_p$. Then, as in the "point hit" method, repeat the procedure but, this time, employ circles of the same size as the resolution boundary circle; placing the centers of these circles at the points of the hit method, observe which structures fall within the boundary of each circle and count the number of hits for each class of structure. Express the number of hits as a percentage of the total number of circles used; for each class, designate as ${}_nV_o$. If the structure is diffuse, it is expected that it could be caught and therefore recorded more often in circles than in hits, so that ${}_nV_p$ would be less than ${}_nV_o$. Hence, the index ${}_nV_p/{}_nV_o$ (≤ 1) is larger the more compact the structure, and is smaller the more diffuse the structure. It is proposed to use this factor to correct for the error in assigning classes to shared grains as a result only of their diffuse shape and not of their radioactivity. Thus, in the formula, to obtain the proportion of all grains shared by classes n, n', n'' which could be attributed to n alone, multiply the factors ${}_nC, {}_{n'}C, {}_{n''}C$ by the corresponding index V_p/V_o , so that the influence of the true grain count for each class will be reduced according to how diffuse its structure is. In practice, for most classes of structures, the index ${}_nV_p/{}_nV_o$ may not be sufficiently different from unity to make much difference; however, for some classes where the structure is spread out, it may differ and, in these instances, the modification will be indicated.

Hence, if the number of grains shared by classes n, n', n'' is designated as ${}_nS_{n',n''}$, and the number which can be attributed to n alone is designated as ${}_nS_{n',n''}$ then

$${}_nS_{n',n''} = {}_nS_{n',n''} \cdot \frac{{}_nC({}_nV_p/{}_nV_o)}{{}_nC({}_nV_p/{}_nV_o) + {}_{n'}C({}_{n'}V_p/{}_{n'}V_o) + {}_{n''}C({}_{n''}V_p/{}_{n''}V_o)} \quad (1)$$

The procedure (still only in theory because every

C is yet unknown) will be to perform this operation for every combination of shared grains. The result will yield the total number of shared grains which can be attributed to every n . In other words,

$${}_nC = {}_nE + \sum {}_nS \quad (2)$$

where $\sum {}_nS$ will take into account every combination of shared grains involving n with one or more of all the other classes of structures.

To make this calculation, start with the first approximation that the ratio of the counts of grains, ${}_nE$, to the sum of exclusive grains, $\sum {}_nE$, is in proportion to the ratio of the true grain counts, ${}_nC$, to the total T_{EM} . Thus, as a first approximation of ${}_nC$, designated as ${}_nC_1$, calculate

$${}_nC_1 = {}_nE \frac{T_{EM}}{\sum {}_nE} \quad (3)$$

In this manner, knowing ${}_nC_1$ (which is used instead of ${}_nC$), ${}_nV_p$, and ${}_nS_{n',n''}$ (for all combinations of classes), the values ${}_nS_{n',n''}$ can be estimated from (1). Then, $\sum {}_nS$ can be inserted into (2) and this would yield a second approximation ${}_nC_2$. If for each class, ${}_nC_1$ is less than 5% different than ${}_nC_2$, then ${}_nC_2$ is accepted as the true ${}_nC$. If there is more than 5% difference in any class, then use ${}_nC_2$ as a second approximation for ${}_nC$ in (1) to find ${}_nC_3$ in (2), and so on. (This type of analysis would lend itself to electronic computer calculation.) Ultimately, the results will yield statistical values for ${}_nC$ (for all classes) which satisfy (1) and (2). The relative content of radioactive label in each class of structure would be $({}_nC/T_{EM}) \times 100$ in units of true grain counts as per cent of total observed.

In order to calculate the relative concentration of radioactive label in the various classes of structures, merely divide ${}_nC/T_{EM}$ by ${}_nV_p$. The values thus obtained, for a given class, are in units of true grain counts (expressed as per cent of the total number of grains) determined per unit volume (expressed as per cent of the total volume of the anatomical region).

In order to compare the content or concentration of radioactive label in a given class of structure under different experimental conditions, such as at various times after administration of the labeled precursor, it is necessary to obtain objective comparisons of T_{EM} under these conditions. For technical reasons, this may be difficult to achieve by using electron microscope radioautographs alone. It requires assurance that values for T_{EM} in

the different conditions are not affected by merely having included smaller or larger areas of the radioautographs for survey or by varying photographic conditions such as exposure and development. In fact, to compare T_{EM} objectively, it is likely to be more feasible to use, in addition, light microscope radioautographs. Here, under uniform photographic conditions, grain counts over larger fields of vision, say, whole cells at various times after administration of labeled precursor, would yield valid relative total grain counts, T_{LM} , which would represent the total content of radioactive label in an entire anatomical region, such as a cell. Then, for each experimental condition, to obtain the corrected value for the content of radioactive label in each class of structure, calculate ${}_nC(T_{LM}/T_{EM})$, as these values in units of true grain counts can be compared with those pertaining to any other class for any of the other experimental conditions. Similarly, to obtain the corrected values for the concentration of label in each class, calculate $({}_nC/{}_nV_p)(T_{LM}/T_{EM})$. The values thus obtained for a given class are in units of true grain counts determined per unit volume (expressed as per cent of the total volume of the anatomical region).

SAMPLE CALCULATIONS

Data for 20 min after tracer fucose- 3H injection into rat is used.

Since the object was to determine the content of radioactive label in each of the thyroid follicular cell structures relative to the whole content of label in the cell, attempts were made to select electron microscope radioautographs illustrating as much of the cell as possible to include the whole of the radioautographic reaction. Because, for the most part, no grains appeared over the basal region of the cells, this made it easy, and attention had only to be confined to the apical portion where whole fields in the electron microscope could be obtained.

According to theory,¹ resolution boundary circles about each silver grain were drawn with radii of 6.7 μ m (based on specifications of electron microscope magnification 30,000, tissue section 0.1 μ m, monolayer of closely packed silver halide crystals of diameter 0.1 μ m). A total of 468 counted grains was used for analysis ($T_{EM} = 468$). From Table II (in the main text), at 20 min after fucose- 3H , exclusive grains were assigned only to

the Golgi apparatus, rough endoplasmic reticulum, and apical vesicles. Ignored in the analysis was each class of structure (such as mitochondria, lysosomes, colloid droplets, and plasma membrane) to which no exclusive grains were assigned (and to which the shared grains assigned happened to be less than 5% T_{EM}). Since the number of exclusive grains assigned to the nucleus was less than 1% of the total, the nucleus was also ignored. Thus, under shared grains, all classes of structures other than Golgi apparatus, rough endoplasmic reticulum, and apical vesicles were designated as "others." Counts of shared grains between Golgi apparatus and "others" were included as exclusive to Golgi apparatus; similarly for rough endoplasmic reticulum and "others," and for apical vesicles and "others." Counts of shared grains between any two of the three classes with exclusive grains and "others" were listed as shared grains only between the two with exclusive grains. Accordingly, see Exhibit A.

Since within a circle more than one structure could be hit, the total number of circle hits (1012) exceeded the total number of point hits (681), even though the numbers employed of points and circles were the same.

From (3), as a first approximation,

$${}_gC_1 = 144 \times \frac{468}{267} = 252,$$

$${}_{rER}C_1 = 97 \times \frac{468}{267} = 170, \text{ and}$$

$${}_{av}C_1 = 26 \times \frac{468}{267} = 46.$$

From (2),

$${}_gC_2 = {}_gE + {}_g{}_{rER} \frac{{}_gC_1({}_gV_p/{}_gV_o)}{{}_gC_1({}_gV_p/{}_gV_o) + {}_{rER}C_1({}_{rER}V_p/{}_{rER}V_o)},$$

so that

$${}_gC_2 = 144 + 118$$

$$\frac{252 \times \frac{9.1}{9.5}}{\left(252 \times \frac{9.1}{9.5}\right) + \left(170 \times \frac{50.7}{47.1}\right)} = 213.$$

EXHIBIT A

(a) exclusive grains (nE) were		
Golgi apparatus (G)	144	(140 strictly exclusive, plus 4 shared with "others" which, as explained in Table II, were organelles without exclusive grains of their own),
rough endoplasmic reticulum (rER)	97	(43 + 54), and
apical vesicles (av)	26	(24 + 2),
	thus, $\sum nE = 267$;	
(b) shared grains ($n_s n'$) were		
G and rER	118	(112) shared between these two classes of structures plus only 6 shared with "others" which, as explained in Table II, were organelles without exclusive grains of their own), and
av and rER	83	(73 + 10),
	thus, $\sigma_{rER}^s = 118$,	
	$rER^s \sigma = 118$,	
	$\sigma_{av}^s rER = 83$,	
	$rER^s \sigma_{av} = 83$.	
(c) The results for the point hit and circle hit methods were (see Table III in the main article)		
	nV_p	nV_o
G	9.1	9.5
rER	50.7	47.1
av	6.2	7.8.

Similarly,

$$rER C_2 = 97$$

$$+ 118 \frac{170 \times \frac{50.7}{47.1}}{\left(170 \times \frac{50.7}{47.1}\right) + \left(252 \times \frac{9.1}{9.5}\right)}$$

$$+ 83 \frac{170 \times \frac{50.7}{47.1}}{\left(170 \times \frac{50.7}{47.1}\right) + \left(46 \times \frac{6.2}{7.8}\right)} = 215$$

and

$$av C_2 = 26 + 83$$

$$\frac{46 \times \frac{6.2}{7.8}}{\left(46 \times \frac{6.2}{7.8}\right) + \left(170 \times \frac{50.7}{47.1}\right)} = 40.$$

Since σC_2 (213) differs by more than 5% from σC_1 (252), (and so do the first and second approximations for rER and av), it is necessary to proceed to higher degrees of approximation. Thus,

$$\sigma C_3 = \sigma E$$

$$+ \sigma_{rER}^s \frac{\sigma C_2 (\sigma V_p / \sigma V_o)}{\sigma C_2 (\sigma V_p / \sigma V_o) + rER C_2 (rER V_p / rER V_o)},$$

so that

$$\sigma C_3 = 144$$

$$+ 118 \frac{213 \times \frac{9.1}{9.5}}{\left(213 \times \frac{9.1}{9.5}\right) + \left(215 \times \frac{50.7}{47.1}\right)} = 200.$$

Similarly,

$$rER C_3 = 97$$

$$+ 118 \frac{215 \times \frac{50.7}{47.1}}{\left(215 \times \frac{50.7}{47.1}\right) + \left(213 \times \frac{9.1}{9.5}\right)}$$

$$+ 83 \frac{215 \times \frac{50.7}{47.1}}{\left(215 \times \frac{50.7}{47.1}\right) + \left(40 \times \frac{6.2}{7.8}\right)} = 232,$$

and

$$av C_3 = 26$$

$$+ 83 \frac{40 \times \frac{6.2}{7.8}}{\left(40 \times \frac{6.2}{7.8}\right) + \left(215 \times \frac{50.7}{47.1}\right)} = 36.$$

Again, since σC_3 (200) differs by more than 5% from σC_2 (213), (and likewise for rER and av),

proceed as follows:

$${}_G C_4 = 144$$

$$+ 118 \frac{200 \times \frac{9.1}{9.5}}{\left(200 \times \frac{9.1}{9.5}\right) + \left(232 \times \frac{50.7}{47.1}\right)} = 196,$$

$${}_{rER} C_4 = 97$$

$$+ 118 \frac{232 \times \frac{50.7}{47.1}}{\left(232 \times \frac{50.7}{47.1}\right) + \left(200 \times \frac{9.1}{9.5}\right)}$$

$$+ 83 \frac{232 \times \frac{50.7}{47.1}}{\left(232 \times \frac{50.7}{47.1}\right) + \left(36 \times \frac{6.2}{7.8}\right)} = 238,$$

and,

$${}_{av} C_4 = 26$$

$$+ 83 \frac{36 \times \frac{6.2}{7.8}}{\left(36 \times \frac{6.2}{7.8}\right) + \left(232 \times \frac{50.7}{47.1}\right)} = 35.$$

Now, ${}_G C_4$ (196) does not differ from ${}_G C_3$ (200) by more than 5%; nor do ${}_{rER} C_4$ (238) and ${}_{rER} C_3$ (232), or ${}_{av} C_4$ (35) and ${}_{av} C_3$ (36). Hence, it is not required to proceed to any higher degree of approximation. In terms of relative content of radioactive label, since ${}_n C = 196 + 238 + 35 = 469$, the Golgi apparatus would contain 196/469 or 42%, the rough endoplasmic reticulum 238/469 or 51%, and the apical vesicles 35/469 or 7%.

To calculate the *relative concentration of radioactive label* in the three structures, divide ${}_n C/T_{EM}$ by ${}_n V_p$, so that for Golgi apparatus it is 42/9.1 or 4.6, for the rough endoplasmic reticulum it is 51/50.7 or 1.0, and for the apical vesicles it is 7/6.2 or 1.2 (Fig. 22 in the main article).

In a light microscope study T_{LM} was found to be 8.1 (in relation to similar counts at other times after fucose- 3H injection). Therefore, the corrected value used to compare the concentration of label in each class with any other class at any time was, for G, $4.6 \times 8.1 = 37$; for rER, $1.0 \times 8.1 = 8.1$; and for av, $1.2 \times 8.1 = 10$, in units of true grain counts per volume of structure as percentage of the total volume of the follicular cell (Fig. 23 in the main article).

REFERENCES

- CARO, L. G. 1962. High resolution autoradiography. *J. Cell Biol.* 15:189.
- CHALKLEY, H. W. 1943. Method for the quantitative morphologic analysis of tissues. *J. Nat. Cancer Inst.* 4:47.
- COIMBRA, A., and C. P. LEBLOND. 1966. Sites of glyco-gen synthesis in rat liver cells as shown by electron microscope radioautography after administration of glucose- 3H . *J. Cell Biol.* 30:151.
- GRANBOULAN, P. 1965. Comparison of emulsion and techniques in electron microscope radioautography. *Symp. Int. Soc. Cell Biol.* 4:43.
- MIURA, T., and V. MIZUHIRA. 1965. Determination of autoradiographic resolutions by 3H - or ^{32}P -labeled RNA phages in electron microscopy. *J. Electron Microsc.* 14:327.
- PELC, S. R. 1963. Theory of electron autoradiography. *J. Roy. Microsc. Soc.* 81:131.
- SALPETER, M. M., and L. BACHMANN. 1965. Assessment of technical steps in electron microscope autoradiography. In *The Use of Radioautography in Investigating Protein Synthesis*. Symposium of the International Society of Cell Biology. C. P. Leblond and K. B. Warren, editors. Academic Press Inc., New York. 4:23.
- SALPETER, M. M., L. BACHMANN, and E. E. SALPETER. 1969. Resolution in electron microscope radioautography. *J. Cell Biol.* 41:1.
- WILLIAMS, M. A. 1969. The assessment of electron microscope autoradiographs. *Advan. Opt. Electron Microsc.* 3:219.