

# RADIOAUTOGRAPHIC VISUALIZATION OF THE INCORPORATION OF GALACTOSE-<sup>3</sup>H AND MANNOSE-<sup>3</sup>H BY RAT THYROIDS IN VITRO IN RELATION TO THE STAGES OF THYROGLOBULIN SYNTHESIS

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## ABSTRACT

Rat thyroid lobes incubated with mannose-<sup>3</sup>H, galactose-<sup>3</sup>H, or leucine-<sup>3</sup>H, were studied by radioautography. With leucine-<sup>3</sup>H and mannose-<sup>3</sup>H, the grain reaction observed in the light microscope is distributed diffusely over the cells at 5 min, with no reaction over the colloid. Later, the grains are concentrated towards the apex, and colloid reactions begin to appear by 2 hr. With galactose-<sup>3</sup>H, the reaction at 5 min is again restricted to the cells but it consists of clumped grains next to the nucleus. Soon after, grains are concentrated at the cell apex and colloid reactions appear in some follicles as early as 30 min. Puromycin almost totally inhibits incorporation of leucine-<sup>3</sup>H and mannose-<sup>3</sup>H, but has no detectable effect on galactose-<sup>3</sup>H incorporation during the 1st hr. Quantitation of electron microscope radioautographs shows that mannose-<sup>3</sup>H label localizes initially in the rough endoplasmic reticulum, and by 1-2 hr much of this reaction is transferred to the Golgi apparatus. At 3 hr and subsequently, significant reactions are present over apical vesicles and colloid, while the Golgi reaction declines. Label associated with galactose-<sup>3</sup>H localizes initially in the Golgi apparatus and rapidly transfers to the apical vesicles, and then to the colloid. These findings indicate that mannose incorporation into thyroglobulin precursors occurs within the rough endoplasmic reticulum; these precursors then migrate to the Golgi apparatus, where galactose incorporation takes place. The glycoprotein thus formed migrates via the apical vesicles to the colloid.

## INTRODUCTION

Thyroid follicles are composed of a single layer of epithelial cells which surround a lumen containing a viscous fluid, the colloid. Cytophotometric analysis of rat follicles demonstrated that thyroglobulin<sup>1</sup> is abundant in the colloid and present in

small quantities in the cells (1). This glycoprotein contains 8-10% carbohydrate covalently linked to polypeptide chains (2-5). The carbohydrate is present in the form of 23 oligosaccharide side-chains, nine of which contain only *N*-acetylglucosamine and mannose, while the others consist of an inner core of *N*-acetylglucosamine and mannose

<sup>1</sup>The following terminology has been adopted: "Thyroglobulin" refers to the completed glycoprotein with a sedimentation coefficient of 17-19S which may or may not be iodinated. "Iodinated thyroglobulin" is restricted to the completed glyco-

protein containing iodine. "Thyroglobulin precursors" refer to incompletely glycosylated molecules due to become thyroglobulin.

to which are attached branches beginning with *N*-acetylglucosamine, followed by galactose and terminating with sialic acid or fucose (6, 7).

The formation of thyroglobulin has been previously studied with the radioautographic technique. Shortly after injection of radioiodine into rats, a radioautographic reaction was observed over the colloid, which is, therefore, the site of iodine uptake into thyroglobulin (8). After injection of radioactive amino acids, the radioautographic reaction was initially found over follicular cells, and, with time, appeared over the colloid as the cellular reaction decreased. It was concluded that the polypeptide components of thyroglobulin are synthesized within the cytoplasm of the cells and later secreted into the colloid (9-11). Examination of radioautographs in the electron microscope after injection of leucine-<sup>3</sup>H revealed that at 10 min the silver grains are located over the rough endoplasmic reticulum (ER) of the follicular cells; by 1 hr the grains are concentrated in the Golgi apparatus, and a few grains are found over apical vesicles; and by 3.5 hr some grains are seen over the colloid (12). Hence, the migratory pathway of thyroglobulin precursors is via the cisternae of the rough ER, the Golgi apparatus, the apical vesicles, and thence into the colloid.

Biochemical studies indicated that the synthesis of thyroglobulin takes place in several stages. Polypeptide subunits of thyroglobulin are formed first; these later aggregate into a noniodinated form of thyroglobulin, which subsequently becomes iodinated (13-25). There is some evidence that the synthesis of the carbohydrate side-chains is a stepwise process which occurs after polypeptide chain synthesis and which is probably completed before iodination. Spiro and Spiro (26) observed that puromycin, which completely inhibited the synthesis of the polypeptides of thyroglobulin, had much less effect on the synthesis of the carbohydrate side-chains from glucose-<sup>14</sup>C. Furthermore, puromycin inhibited the labeling of sugars located near the polypeptide chains far more than that of sugars situated farther away. Herscovics (25) showed that mannose, which is closer to the polypeptide chains than galactose, is incorporated into subunit precursors of thyroglobulin at about the time of their synthesis, whereas galactose is incorporated over 30 min later near the time of aggregation of subunits to noniodinated thyroglobulin. Since the polypeptide precursors were known to

migrate from rough endoplasmic reticulum through Golgi apparatus to colloid (12), the question arose whether mannose and galactose are incorporated at different subcellular locations. The radioautographic technique was then used to examine thyroid lobes incubated *in vitro* for various periods of time with tritium-labeled mannose, galactose, and leucine. This was repeated in some cases after addition of puromycin. Examination of the radioautographs clarified the role played by cell organelles in the elaboration of the carbohydrate side-chains of thyroglobulin.

## MATERIALS AND METHODS

### *Incubation of Thyroid Lobes*

Male Sherman rats kept on a diet of Purina Chow were used. The larynx and upper part of the trachea with the attached thyroid gland were removed under ether anesthesia and immediately immersed in ice-cold saline which had been previously gassed with oxygen for 15 min. The thyroid lobes were then dissected free of the trachea, and traces of fat and connective tissue were removed, while the tissues were kept submerged in cold oxygenated saline.

In preliminary light microscope experiments, the lobes were submerged in bicarbonate-buffered Krebs-Ringer solution in equilibrium with an atmosphere of O<sub>2</sub>/CO<sub>2</sub> (95:5) (27). Incubation was carried out in a shaking water bath at 37°C. Under these conditions, signs of cell degeneration in the central part of the lobes were apparent within 15-30 min, and central necrosis of the lobes became more marked with time. These findings were interpreted to indicate an inadequate supply of oxygen to the inner part of the lobe. The following incubation technique was, therefore, designed to increase the availability of oxygen by limiting the solution surrounding the lobes to a thin film.

In all the experiments described in this paper, strips of Whatman No. 40 filter paper previously wetted with the appropriate medium were placed horizontally on glass rods supported at each end on the sides of an open box. The lobes were suspended in small holes cut in the filter paper and then flooded with medium. The box was placed in a desiccator kept in a 37°C incubator. For maintaining the correct atmosphere and maximum humidity, a mixture of O<sub>2</sub>/CO<sub>2</sub> (95:5) was continuously bubbled through a solution of 0.25 M sodium bicarbonate placed at the base of the desiccator. Gassing was started at least 30 min before the beginning of incubation. The use of this technique reduced significantly the degree of central necrosis, but did not completely eliminate it.

### Light Microscope Radioautography

Thyroid lobes from 250-g rats were incubated with *L-leucine-4,5-T* (specific activities, 23.5 c/mmole or 14.7 c/mmole; Radiochemical Centre, Amersham, England) at a concentration of 10  $\mu\text{c/ml}$  of Krebs-Ringer bicarbonate. As indicated in the legends of the figures, both continuous and pulse-labeling techniques (described under Electron Microscope Radioautography) were used. After incubation, the lobes were fixed in Bouin's fluid for 24 hr and all the material for one experiment was embedded in paraffin in a single block. Sections were cut at 5  $\mu$  and stained with either hematoxylin and eosin, or the periodic acid-Schiff reaction and hematoxylin before being coated with Kodak NTB2 emulsion for radioautography. After exposure, radioautographs were developed in Kodak D-170 and examined qualita-

tively. The latter were first preincubated in the absence of puromycin or maintained in ice-cold saline, and then incubated with the labeled substrate.

### Electron Microscope Radioautography

Four experiments with pulse-labeling were carried out (Table I). The lobes were first incubated in Krebs-Ringer bicarbonate containing labeled substrate for only a short period of pulse-labeling, generally 5 min. At the end of this pulse, the lobes were washed with a large volume of "chase" medium consisting of Medium 199 with Earle's base (Baltimore Biological Laboratory, Baltimore) to which the appropriate unlabeled substrate had been added at a concentration of 5 mM. The lobes were then transferred to a new strip of filter paper previously wetted with chase medium, and flooded with more

TABLE I  
Incubating and Processing Procedures for Rat Thyroid Lobes Subsequently Used for Quantitative Electron Microscopy

Expt. No.	Weight of rats $\pm 25$ g	Substrate		Duration of pulse	Additives to "chase"	Durations of incubation (pulse + chase)	Fixation
		Nature and specific activity	Concentration				
1	100	Mannose- <sup>3</sup> H (700 $\mu\text{c/mmole}$ )	650 $\mu\text{c/ml}$	5	Mannose (5 mM), sodium pyruvate (10 mM)	15-240, 4%	paraformaldehyde
2	100	Mannose- <sup>3</sup> H (554 $\mu\text{c/mmole}$ )	400 $\mu\text{c/ml}$	5	Mannose (5 mM), sodium pyruvate (10 mM)	5-240, 2.5%	glutaraldehyde
3	100	Galactose- <sup>3</sup> H (1 c/mmole)	400 $\mu\text{c/ml}$	10	—	10-180, 2.5%	glutaraldehyde
4	150	Galactose- <sup>3</sup> H (1 c/mmole)	400 $\mu\text{c/ml}$	5	Galactose (5 mM)	5-180, 2.5%	glutaraldehyde

tively. Strong radioautographic reactions were observed within a 3-wk exposure period.

Similar experiments were carried out with either *D-mannose-1-T* (specific activities, 700 mc/mmole or 554 mc/mmole) or *D-galactose-1-T* (specific activities, 1 c/mmole or 1.7 c/mmole; Radiochemical Centre, Amersham, England) at a concentration of 100  $\mu\text{c/ml}$ . Lobes simultaneously incubated in leucine-<sup>3</sup>H were used as controls.

*Puromycin* dihydrochloride (Nutritional Biochemical Corporation, Cleveland) at a concentration of 125  $\mu\text{g/ml}$  of bicarbonate-buffered Krebs-Ringer solution was used to inhibit thyroglobulin synthesis. Thyroid lobes were first preincubated at 37°C in a medium containing puromycin for a period of 20 min. They were then subjected to labeling with leucine-<sup>3</sup>H, mannose-<sup>3</sup>H, or galactose-<sup>3</sup>H in the presence of puromycin and processed alongside con-

chase medium. In some cases, fresh chase medium was added every half-hour.

In the experiments with mannose-<sup>3</sup>H, sodium pyruvate was added to all media at a concentration of 10 mM to reduce the conversion of this substrate into amino acids (25).

After fixation in 2.5% glutaraldehyde for 3 hr followed by phosphate buffer wash overnight, or in 4% paraformaldehyde for 5 hr, the tissues were placed in 1% buffered osmium tetroxide for 2 hr. The tissues were then dehydrated in acetone and embedded in Epon, and the blocks were cut on a Porter-Blum microtome. For radioautography, silver-gold sections were transferred to celloidin-coated slides (28) and coated with dilute Ilford L4 emulsion by a method which insures a single uniform layer of closely packed silver bromide crystals (29). After exposure, the emulsion was developed in Kodak

D-19b and the area containing sections was floated onto distilled water for transfer to electron microscope grids. The sections were then stained for quantitative work in a saturated solution of uranyl acetate in 50% alcohol for 10 min, followed by 30 min in lead citrate (30). Scanning was carried out in either a Siemens Elmiskop I or a Hitachi microscope. Photographs of follicular cells were systematically taken in rows so as to avoid bias.

### *Analysis of Reactions in Electron Microscopic Radioautographs*

Before one attempts to relate the silver grains observed in the photographs to the organelles from which the radioactivity originates, three factors require consideration:

**BACKGROUND FOG:** The number of grains due to fog in preparations of this type was very low (less than one grain per 16 square microns, on the average). The rare grains arising from this factor should be distributed at random.

**POSSIBILITY OF BINDING OF FREE MANNOSE-<sup>3</sup>H AND GALACTOSE-<sup>3</sup>H:** Binding of free substrate to the fixed tissue was found to be negligible under present conditions, as shown by the following experiment. Thyroid lobes were incubated for 10 min at 4°C with labeled substrate. At this temperature, the labeled substrates penetrate the lobes with little incorporation into macromolecules, so that any observed radioautographic reaction can be ascribed to binding of the free substrate. After fixation and radioautography, the lobes were examined in the light microscope. With glutaraldehyde, the grain concentration was high with leucine-<sup>3</sup>H (45 times background) and glucosamine-<sup>3</sup>H (22 times background), and was near background with both galactose-<sup>3</sup>H and mannose-<sup>3</sup>H. In the case of paraformaldehyde fixation, the grain concentration was near background with all substrates. It was, therefore, concluded that after fixation with glutaraldehyde binding was high with free leucine-<sup>3</sup>H (see 31-33), but not detectable with free galactose-<sup>3</sup>H or mannose-<sup>3</sup>H; in the case of paraformaldehyde fixation, no binding was detected with any of these substrates.

**RESOLUTION OF THE TECHNIQUE:** Because a  $\beta$ -ray may travel more than 1  $\mu$  from its source, it does not necessarily hit the silver crystal which lies immediately over it. And even when it does, a single undeveloped crystal (0.12  $\mu$ ) or developed silver grain (0.27  $\mu$ ) often overlaps several structures. As a result, the majority of grains in radioautographs of the thyroid follicular cell are attributable to more than one intracellular structure. The radioautographs were, therefore, analyzed as follows. A circle with a radius of 2250 Å was drawn around the assumed center of each grain. According to unpublished

calculations of N. J. Nadler based on the use of 0.1- $\mu$  thick sections and a closely packed monolayer of spherical silver bromide crystals with a diameter of 0.1  $\mu$  (Ilford crystals have a mean diameter of 0.128  $\mu$ ), this circle would contain the source of <sup>3</sup>H radiation in the tissue with 95% probability. This conclusion was not in accord with the data of Bachmann et al. indicating that the probability of a silver grain being within 2250 Å of a point source was only 50% (Fig. 9 of reference 34), but was in fair agreement with the data of Caro (35) indicating that, with a 600-Å phage as radioactive source, 92% of the grains were located within a distance of 2250 Å from the edge of the source (his Fig. 6), and with *Bacillus subtilis*, 98% of the grains were within that same distance (his Fig. 8).

In practice, the 2250-Å circles were cut in a clear plastic sheet to be used as stencil, their size being proportional to the magnification, with a 6.7-mm radius for the magnification of 30,000 used in most of this work. This stencil was placed over pictures of radioautographs so that a silver grain was located centrally within a circle. The structures in the circle were then identified. A points system was used whereby a single structure occupying a circle received one point; when two, three, or four structures were present, each received one-half, one-third, or one-fourth of a point, respectively. The number of points received by any given structure was calculated and expressed as the percentage of the total number of grains counted.<sup>2</sup>

The following structures were looked for within the "95% probability" circles: *rough ER* (including cisternae and associated ribosomes; free ribosomes were ignored); *Golgi apparatus* (including saccules and associated smooth vesicles); *apex* (including apical vesicles as well as the apical plasma membrane, since most apical vesicles are located along this plasma membrane where they are often hidden by grains); *plasma membrane* (including only lateral and basal cell walls); *lysosomes* (including all dense bodies conforming to criteria laid down previously; 36); *mitochondria*; and *colloid droplets*. For each time interval of each experiment, 200-300 grains were counted in randomly photographed material. The results obtained are referred to as "uncorrected grain distribution." Repeated counts of 200 grains in different photographs from the same sample

<sup>2</sup> Some of the data were also recorded by giving each organelle within the circle a whole point irrespective of how many organelles were present. The results obtained by this method were substantially similar to those reported with the method used in the article, which was thought to be more useful in that it automatically recorded the number of grains counted.

yielded percentage distribution in which the differences were less than  $\pm 3\%$ .

The distribution of grains obtained by the method just described does not provide an exact picture of the distribution of radioactivity among the various subcellular structures. The structures within the cell may be roughly divided into two types: one type, e.g. the nucleus, is relatively compact and does not intermingle with other structures and, therefore, most of the overlying silver grains truly originate there. However, the other type, e.g. rough ER, is dispersed and intermingles with other structures, so that reactions recorded for it do not necessarily originate in it. Some method had to be devised to eliminate the spurious counts attributed to a non-labeled structure as a result of its close association with a labeled one. The method used is based on the assumption that since most of the cytoplasmic structures of the follicular cell approach the dispersed type, they are randomly distributed, so that any spurious counts should also be randomly distributed.

TABLE II  
Percentage Areas Occupied by Cytoplasmic Structures in Thyroid Follicular Cells

	%
Rough endoplasmic reticulum	50
Golgi apparatus	10
"Apex"	15
"Plasma membrane"	7
Lysosomes	2
Mitochondria	14
Colloid droplets	2

Under these conditions, the grain distribution resulting from these spurious counts should be proportional to the area occupied by each structure. This conclusion would also apply to background fog and to any binding of free substrate. The percentage area of the cytoplasm occupied by each of the structures was, therefore, determined and used as an estimate of the "random grain distribution." This was done by placing a stencil containing a large number of "95% probability" circles at random over photographs of the cytoplasm in which there was no radioautographic reaction. These photographs were obtained from the same blocks that were used for radioautography. The contents of 500 circles were analyzed by the same points system used for the analysis of grain reactions, and the average of results from four different experiments were recorded in Table II. Assessed in this way, the area of organelles was constant within a limit of  $\pm 3\%$  throughout the four experiments.

By use of the present method, only those reactions which are different from random distribution can

unequivocally be considered as significant. The difference was determined by deducting the "random grain distribution" (Table II) from the "uncorrected grain distribution" (Figs. 19, 21, 26, 28). The result was referred to as "corrected grain distribution" (Figs. 20, 22, 27, 29).

The graphs of "corrected grain distribution" represent neither actual grain concentration, nor actual radioactivity, but indicate solely when a grain reaction is significant and, therefore, attributable to a particular structure. Since the correction is based on random distribution of structures, it is too severe, especially in the case of Golgi apparatus which is less randomly distributed than the other structures. (It may be added that low concentrations of radioactivity, e.g. the radioactivity due to the formation of sedentary intracellular proteins, may go undetected.)

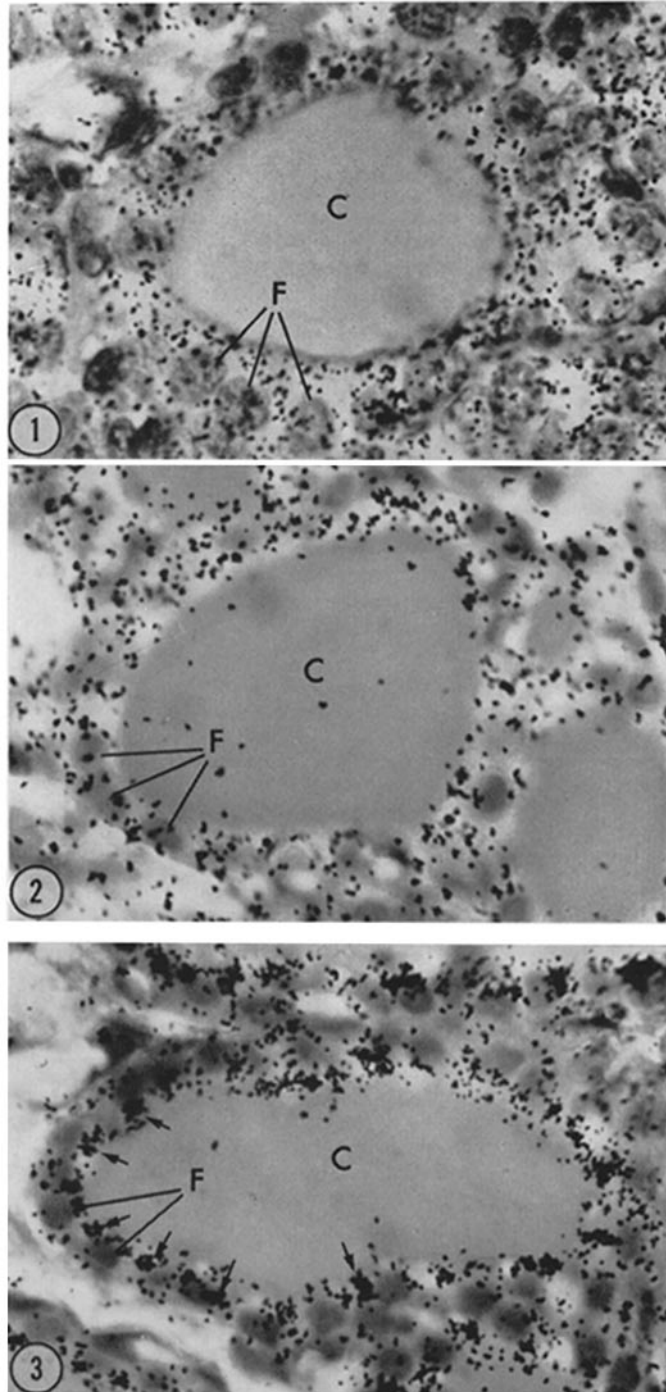
## RESULTS

### *Entry of Labeled Substrates into the Lobes*

That the three labeled substrates (leucine- $^3\text{H}$ , mannose- $^3\text{H}$ , and galactose- $^3\text{H}$ ) reached the center of the incubated thyroid lobes was shown by the fact that after incubation at  $37^\circ\text{C}$  for 5 min with either one of the three substrates, fixation in Bouin's and radioautographic overexposure of the preparations, grain reactions were observed over thyroid cells throughout the lobe, thus indicating that the three labeled substrates did not only enter, but were incorporated by all thyroid cells. It may be added that, following a 5-min incubation in the cold ( $4^\circ\text{C}$  with leucine- $^3\text{H}$  or glucosamine- $^3\text{H}$ ), no reaction was present when fixation was in Bouin's, but when fixation was in glutaraldehyde—a fixative which binds both free leucine and glucosamine to tissues—an even reaction was observed throughout. These various observations demonstrated that labeled precursors diffused throughout the lobes within 5 min.

### *Incorporation of Labeled Substrates as seen by Light Microscopy*

Because of the better survival of the two or three peripheral rows of follicles, they were used for the observations recorded in the present paper. Grain reactions were evenly distributed over all the cells of any given follicle, but varied in intensity from follicle to follicle, in agreement with previous observations in vivo with  $^{125}\text{I}$  (37) and leucine- $^3\text{H}$  (12). In spite of this individual variation among follicles, clear-cut differences were



FIGURES 1-3 Light microscope radioautographs of thyroid lobes of rats 5-10 min after incubation in Krebs-Ringer bicarbonate containing labeled substrate. Stained with hematoxylin and eosin. Each figure shows a thyroid follicle with the colloid in the center (*C*) and follicular cells around it; the letters *F* point to nuclei of follicular cells.  $\times 1100$ .

FIGURE 1 After 5 min of incubation in *leucine-<sup>3</sup>H* the silver grains indicative of a radioautographic reaction are distributed diffusely over the follicular cells and there are none over the colloid.

FIGURE 2 After 10 min of incubation in *mannose-<sup>3</sup>H*, the silver grains are again diffuse over the cells. The few grains seen over the colloid in this figure show the possible extent of the background fog, but do not indicate a positive reaction (see also Fig. 12).

FIGURE 3 After 5 min of incubation in *galactose-<sup>3</sup>H*, the silver grains are again restricted to the follicular cells, but are arranged in small groups located in the apical cytoplasm (small arrows). The rare grains seen over the colloid are again due to the background fog.

observed between the behavior of the labels from leucine-<sup>3</sup>H and mannose-<sup>3</sup>H on the one hand, and the behavior of the label from galactose-<sup>3</sup>H on the other.

5 min after the onset of incubation in leucine-<sup>3</sup>H, grains were located over thyroid follicular cells but not over colloid (Fig. 1). The distribution of grains over each cell was diffuse, and there was no detectable localization over any particular area or structure within the cell. This grain distribution was unchanged at 30 min (Fig. 4). By 1–2 hr, a concentration of grains towards the cell apex was increasingly apparent. At 2 hr, reactions over the colloid were noted for the first time in a few follicles. At later times (Fig. 5), colloid reactions were observed over more and more follicles and their intensity increased, but at 3 and even 4 hr the majority of follicles still did not exhibit colloid reactions.

The grain reaction in thyroid lobes incubated in mannose-<sup>3</sup>H was not detectably different in distribution or timing from that described for leucine-<sup>3</sup>H. After short incubations in mannose-<sup>3</sup>H, there was a diffuse grain reaction over follicular cells (Figs. 2, 6). By 1–2 hr, there was visible apical concentration. The first colloid reactions were apparent by 2 hr and, as with leucine-<sup>3</sup>H, their number and intensity increased with time (Fig. 7).

In lobes incubated in galactose-<sup>3</sup>H for 5 min, the grain reaction was also restricted to follicular cells, but grains were not diffusely distributed, since they made up small groups in the supranuclear region (Fig. 3). Furthermore, by 30 min, silver grains were present over the colloid in a number of follicles (Fig. 8). They were seen over the colloid of all follicles by 1–3 hr (Fig. 9).

### *Effect of Puromycin*

In thyroid lobes preincubated with puromycin and then incubated with leucine-<sup>3</sup>H in the presence of puromycin, there was an immediate inhibition of the uptake of label. After a 5-min incubation, the grain concentration was negligible compared to that in controls not exposed to puromycin (Figs. 10, 11). After several hours of incubation, the inhibition persisted, but appeared to be less complete. Similarly, the uptake of mannose-<sup>3</sup>H label was immediately and almost completely inhibited by puromycin (Figs. 12, 13). In the case of galactose-<sup>3</sup>H, however, puromycin did not produce an immediate inhibition of incorporation of the

labeled substrate, since after 5 and 30 minutes of incubation the concentration of grains was similar to that of the controls. Even at 1 hr (Figs. 14, 15), the grain concentration was only slightly less than in controls. After this time, the degree of inhibition progressively increased.

### *Electron Microscope Radioautography*

**MANNOSE-<sup>3</sup>H:** After 5 min of incubation in mannose-<sup>3</sup>H, most of the silver grains were associated with the rough ER (Fig. 16). It was not possible to determine whether these grains were located over the membranes or the content of the cisternae. In one of the samples taken at 15 min, however, some of the cisternae of the rough ER were rather wide, and a minority of the grains was attributable to radioactivity originating from their content (Fig. 17). By 1–2 hr, a few silver grains were apparent over the Golgi apparatus (Fig. 18), later over apical vesicles, and finally over the colloid.

The uncorrected grain distribution in the cytoplasm of the thyroid follicular cell after different periods of incubation (Figs. 19, 21) was fairly similar in the two experiments. There was a high initial grain concentration over the rough ER which gradually diminished throughout the experiment. The Golgi reaction was low at first, but increased with time, reaching a peak at 2 hr, while the apex reaction remained low until 1–2 hr, after which time it increased. The reactions over mitochondria, plasma membrane, and other structures remained steady at a low level. The nuclear reactions varied widely from lobe to lobe in an irregular manner, but were not included in the calculations.

In order to establish which of these grain concentrations represented significant reactions, the random grain distribution (Table II) was subtracted from the uncorrected grain distribution as indicated under Methods. In experiment 1, the corrected grain distribution thus obtained (Fig. 20) showed that at 15 min the only significant reaction was that of the rough ER, but by 1.5 hr the Golgi reaction reached a significant level. This was followed by the appearance of an apex reaction at 4 hr. Throughout the experiment, the rough ER reaction kept on declining. In experiment 2 (Fig. 22), the rough ER reaction was again the only significant one at early times, and decreased after 30 min. Meanwhile, a significant Golgi reaction appeared and reached a maximum by 2 hr. The apex reaction was strong at 4 hr.

Hence, in spite of differences between the two experiments, both of them showed an exclusive reaction in the rough ER at first, a Golgi peak at 2 hr, and a subsequent apex reaction.

**GALACTOSE-<sup>3</sup>H:** After 5 or 10 min of incubation with galactose-<sup>3</sup>H some silver grains seemed to be over the rough ER, but in contrast to the observations made with mannose-<sup>3</sup>H, there was already a significant Golgi reaction (Fig. 23). Soon afterwards a decline occurred in the number of grains located over the Golgi apparatus, coinciding with the early appearance of increasingly large numbers of grains over apical vesicles and cell surface (Fig. 24). Later, grains were present over the colloid, a reaction which increased in intensity with time (Fig. 25). At all times, silver grains were seen over the rough ER.

The uncorrected grain distribution (Figs. 26, 28) showed that about 40% of grains were associated with the rough ER but, unlike the mannose-<sup>3</sup>H experiments, this reaction did not decrease with time. The mitochondrial and plasma membrane reactions also remained fairly constant throughout the incubation. In contrast, the grain reaction over the Golgi apparatus was high at 5 and 10 min and declined rapidly thereafter, whereas the reaction over the apex was low initially, but increased very rapidly during the

1st hr. Nuclear reactions were usually low and were not included.

In graphs showing the corrected grain distribution for the two experiments (Figs. 27, 29), the initial significant grain reaction was restricted to the Golgi apparatus, while the apex reaction became significant by 20–30 min. The only other significant reaction was that over lysosomes at 3 hr (Fig. 27). On the whole, there was close agreement between the two experiments in the timing of the various events, since in both an early maximum was observed in the Golgi apparatus and by 1 hr a peak appeared at the apex, whereas the grain distribution over rough ER never exceeded the random distribution value.

## DISCUSSION

### *Secretion of Mannose-<sup>3</sup>H and Galactose-<sup>3</sup>H Label into the Colloid*

Incubation of rat thyroid lobes in vitro in medium containing leucine-<sup>3</sup>H resulted in a diffuse radioautographic reaction over follicular cells at early times (Figs. 1, 4) and over the colloid by 2 or more hr (Fig. 5). The sequence of events was similar to that obtained with leucine-<sup>3</sup>H in vivo (10–12); thyroid follicles were, therefore, functional under the in vitro conditions used, at least

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FIGURES 4–9 Light microscope radioautographs of rat thyroid lobes pulse-labeled for 5 min in Krebs-Ringer bicarbonate containing labeled substrate, and subsequently maintained in vitro for between 30 min and 4 hr in medium containing unlabeled substrate. Staining of figs. 4–6 was with periodic acid-Schiff-hematoxylin and staining of figs. 7–9 with hematoxylin and eosin.  $\times 920$ .

FIGURE 4 Thyroid follicle, 30 min after incubation in leucine-<sup>3</sup>H. The silver grains are restricted to the follicular cells.

FIGURE 5 Thyroid follicle, 4 hr after incubation in leucine-<sup>3</sup>H. In addition to a few grains left over the cells, there is now a pronounced reaction over the colloid.

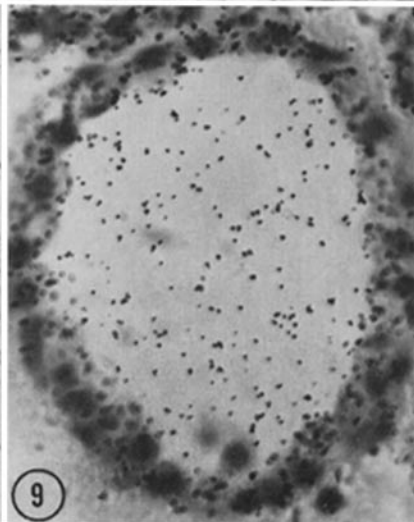
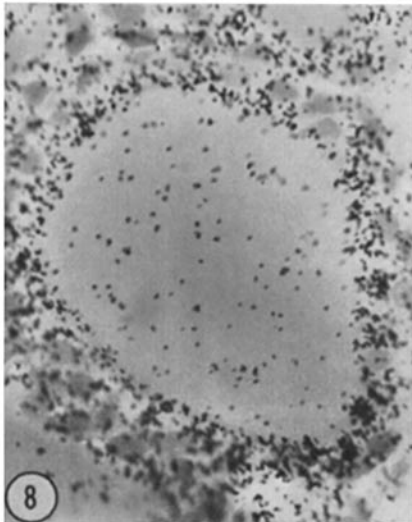
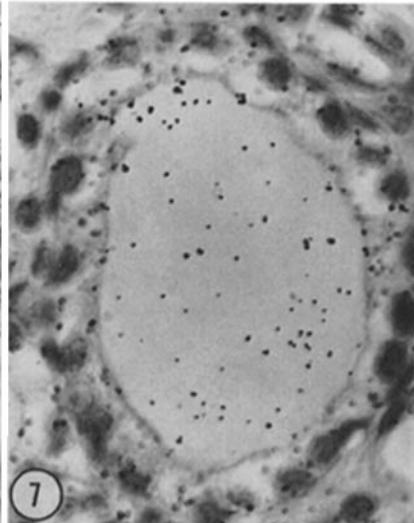
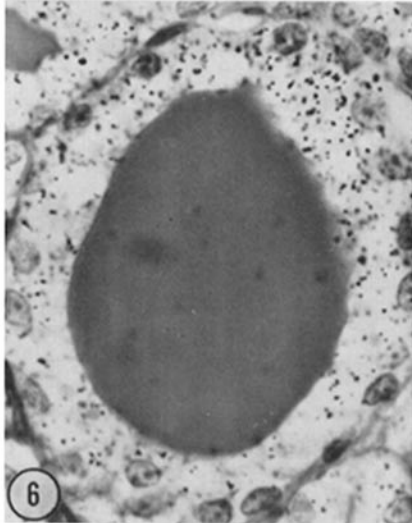
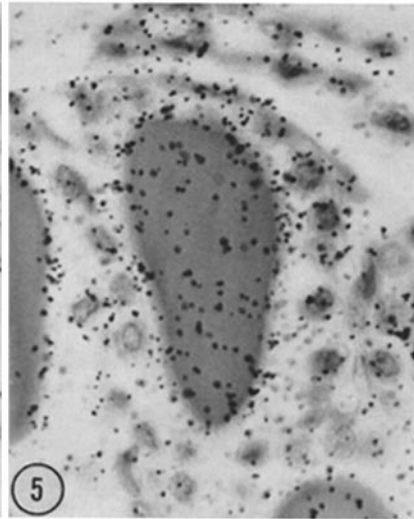
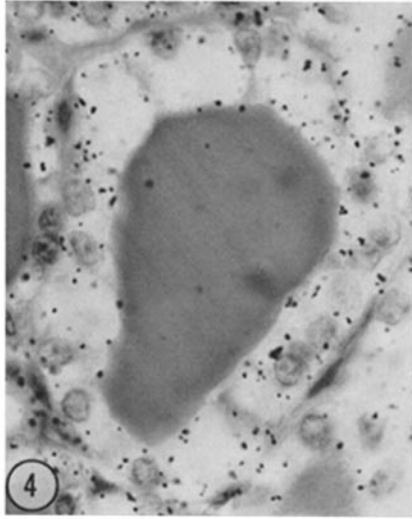
FIGURE 6 Thyroid follicle, 30 min after incubation in mannose-<sup>3</sup>H. The silver grains are restricted to the follicular cells, as after leucine-<sup>3</sup>H incubation.

FIGURE 7 Thyroid follicle, 4 hr after incubation in mannose-<sup>3</sup>H. In addition to a weak cell reaction, there is now a definite colloid reaction.

FIGURE 8 Thyroid follicle, 30 min after incubation in galactose-<sup>3</sup>H. In contrast to leucine-<sup>3</sup>H and mannose-<sup>3</sup>H, there is a marked reaction over the colloid of some follicles at this time.

FIGURE 9 Thyroid follicle, 3 hr after incubation in galactose-<sup>3</sup>H. The colloid reactions are intensified.





in respect to leucine incorporation. On incubation with *mannose-<sup>3</sup>H*, silver grains again appeared first over the cells (Figs. 2, 6) and by 2 or more hr over the colloid (Fig. 7), so that the timing of the migration of label was identical with that of leucine-<sup>3</sup>H. Furthermore, puromycin, an inhibitor of protein synthesis which is said to cause the detachment of incomplete polypeptide chains from ribosomes (38), prevented the uptake of mannose-<sup>3</sup>H as well as that of leucine-<sup>3</sup>H (Figs. 10-13), indicating that the incorporation of mannose-<sup>3</sup>H label is dependent on protein synthesis *de novo*. These conclusions are in line with biochemical observations (25) which showed that initially both labeled leucine and mannose were incorporated into thyroglobulin precursors (3-8S and 12S), and that, in each case, their incorporation could be inhibited by puromycin. Later, these precursors were transformed into uniodinated thyroglobulin (17-18S).

Since the label of mannose behaved like that of leucine, it was possible that the mannose label had been converted to amino acids. However, previous work (25) and unpublished results indicate that under the present conditions of incubation with mannose-<sup>14</sup>C there is little conversion of

label to amino acids and that about 75% of the radioactivity in thyroid proteins is present as mannose. Hence, most of the label observed in radioautographs must have been in the form of mannose. It was, therefore, concluded that mannose itself is incorporated into thyroglobulin precursors at the same time as, or very soon after, leucine.

With *galactose-<sup>3</sup>H*, the early reaction over follicular cells was restricted, in light microscope radioautographs, to small areas next to the nucleus (Fig. 2); and the first reactions over the colloid were detected by 30 min, as compared with 2 hr in the case of leucine-<sup>3</sup>H or mannose-<sup>3</sup>H. Consequently, the completion of thyroglobulin secretion (as indicated by the colloid reactivity) requires 2 hr from the time of incorporation of leucine and mannose, but only 30 min from the time of incorporation of galactose. Therefore, galactose must be incorporated into thyroglobulin precursors about 1.5 hr after mannose. This conclusion received additional support from the fact that inhibition of protein synthesis with puromycin had little effect on galactose incorporation during the 1st hr of incubation (Fig. 15), but was increasingly effective at later times. The delay in the inhibition

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FIGURES 10-15 Light microscope radioautographs of rat thyroid lobes incubated in *puromycin* for 20 min and later in Krebs-Ringer bicarbonate containing labeled substrate in addition to puromycin. In order to assess the degree of inhibition of incorporation of substrate, each reaction is compared to that of a control processed in an identical manner except that puromycin was not present. The staining of Figs. 10 and 11 was in periodic acid-Schiff-hematoxylin, and that of Figs. 12-15, in hematoxylin and eosin.  $\times 920$ .

FIGURE 10 Control thyroid follicle, 5 min after incubation in *leucine-<sup>3</sup>H*.

FIGURE 11 Thyroid follicle from a lobe incubated for 20 min in puromycin and subsequently transferred for 5 min to medium containing *leucine-<sup>3</sup>H*. Comparison with Fig. 10 indicates that incubation in puromycin has inhibited the uptake of label by this lobe.

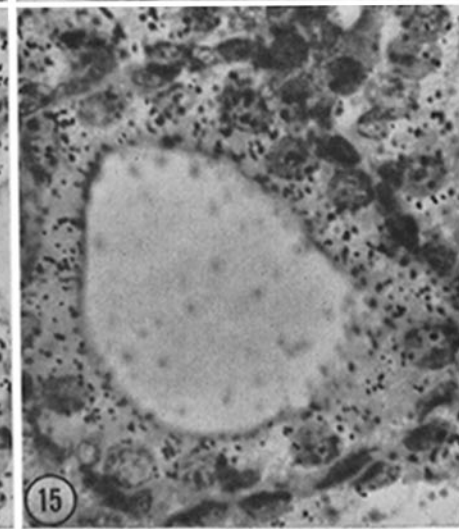
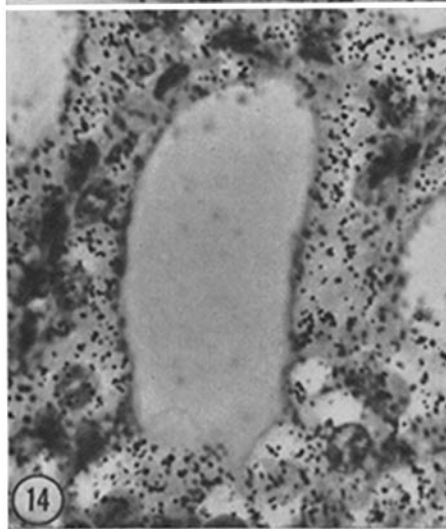
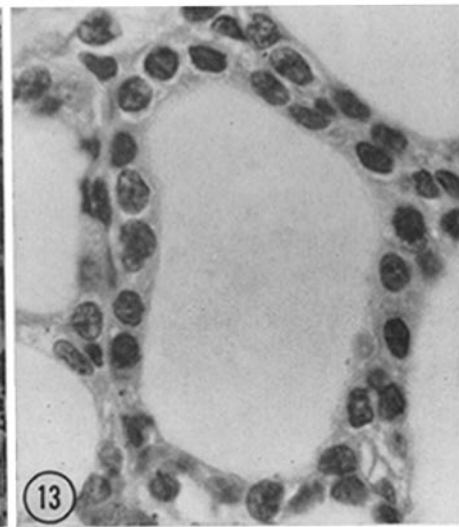
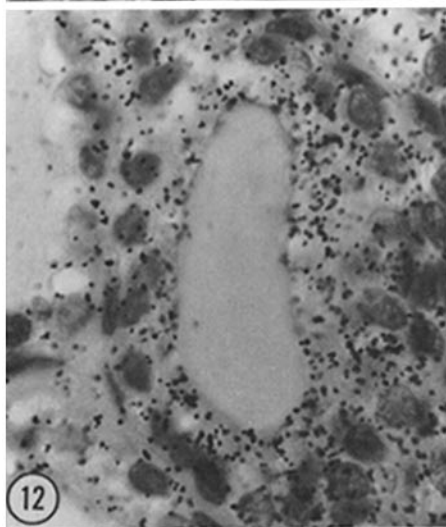
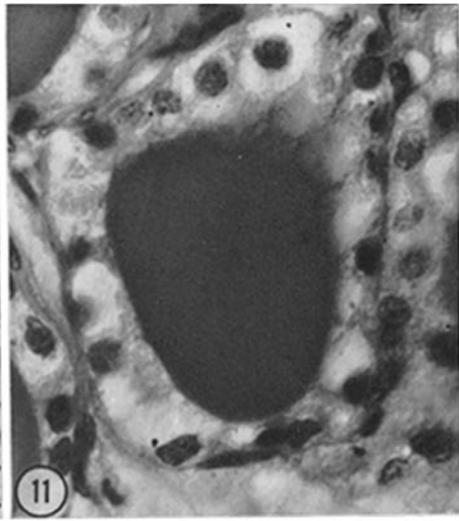
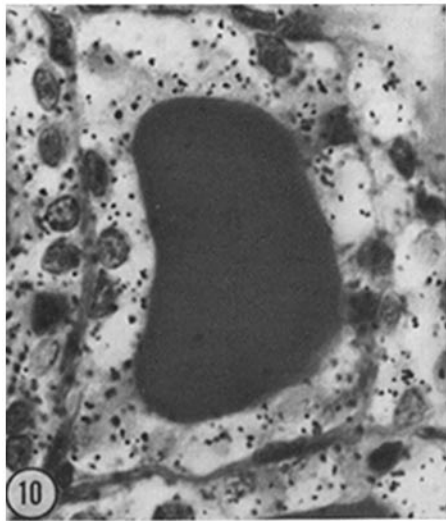
FIGURE 12 Control thyroid follicle after 5 min of incubation in *mannose-<sup>3</sup>H*. The reaction is restricted to the cells.

FIGURE 13 Thyroid follicle from a lobe incubated in puromycin and then for 5 min in medium containing *mannose-<sup>3</sup>H*.

As in the case of leucine-<sup>3</sup>H, the puromycin has inhibited the incorporation of label.

FIGURE 14 Control thyroid lobe continuously labeled in *galactose-<sup>3</sup>H* for 1 hr. There is a strong reaction over cells and a very weak one over the colloid (the silver grains are out of focus).

FIGURE 15 Thyroid follicle from a lobe incubated in puromycin and then continuously labeled with *galactose-<sup>3</sup>H* for 1 hr in the presence of puromycin. When compared to Fig. 14, it is apparent that little or no inhibition has been caused by puromycin.



of galactose incorporation corresponds roughly to the 1.5 hr taken by thyroglobulin precursors to go from the site of mannose incorporation to that of galactose. Biochemical analysis of the distribution of radioactivity in thyroid protein fractions after incubation of thyroid lobes in galactose-<sup>14</sup>C (25) also supports the view that galactose is added at a later stage of thyroglobulin synthesis than mannose; within 10 min the label of galactose-<sup>14</sup>C was present in uniodinated thyroglobulin (17–18S), whereas label from leucine-<sup>3</sup>H and mannose-<sup>14</sup>C was restricted to thyroglobulin precursors (3–8S and 12S) at this time. It may be added that after 30 min of incubation, at least 78% of the label from galactose-<sup>14</sup>C in the 17–18S fraction was recovered as galactose (25). Finally, puromycin had no effect on galactose-<sup>14</sup>C incorporation into the 17–18S fraction at 30 min and only a slight effect at 1 hr (25). These findings are in agreement with the conclusion that the incorporation of galactose into thyroglobulin precursors takes place approximately 1.5 hr after the incorporation of mannose.

#### *Intracellular Incorporation and Migration of Mannose-<sup>3</sup>H and Galactose-<sup>14</sup>C*

The electron microscope experiments had two main purposes. The first was to establish the intracellular locations at which mannose and galactose were incorporated into thyroglobulin. This was

done by locating the label at very early time intervals (5–15 min), to minimize the chance of subsequent migration. The second function of the experiments was to identify the pathway of the labels from their sites of incorporation to their final destination in the colloid.

In the case of mannose-<sup>3</sup>H, at the earliest times of incubation all the radioactivity in the cytoplasm was located in the rough ER (Figs. 16, 19–22), which is, therefore, considered to be the actual site of incorporation of mannose-<sup>3</sup>H into thyroglobulin precursors. Because of limitations of resolution, we could not demonstrate conclusively whether mannose incorporation took place on the polysomes or in the cisternae. Nevertheless, in one of the animals, some of the label was observed in the cisternae by 15 min (Fig. 17). Biochemically, it has been shown that mannose-<sup>14</sup>C labeled the microsomes, but not their polysome fraction (39). Hence, it seemed more likely that the incorporation of mannose took place within the cisternae after completion of polypeptide synthesis.

Changes observed in the distribution of mannose label after 15 min indicated the pathway of migration of the label in follicular cells. A gradual decline of the grain reaction in the rough ER (Figs. 20, 22) coincided with an increasing reaction over the Golgi apparatus. These events took place within the first 2 hr. During that period, the electron microscope revealed that there was no

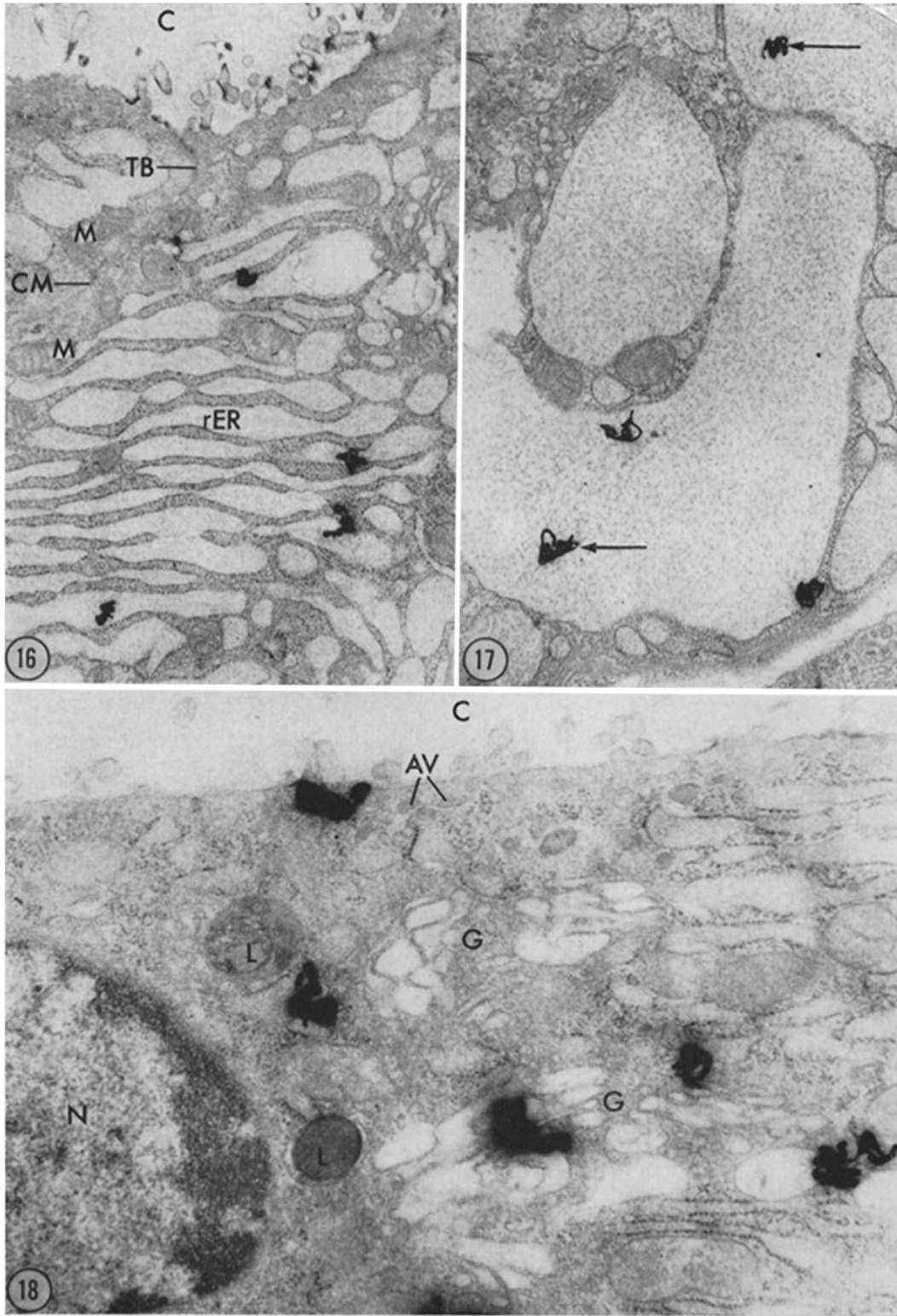
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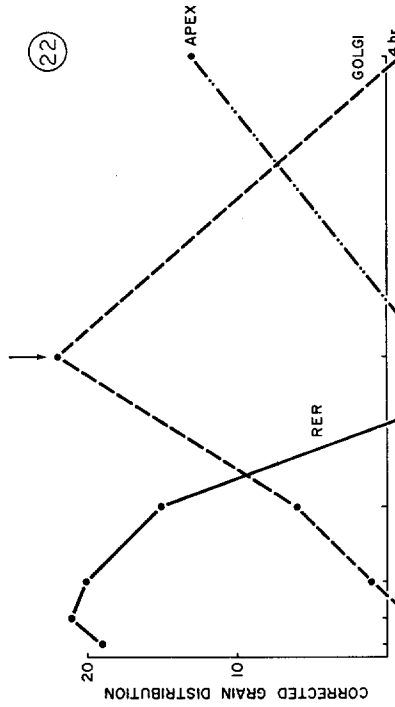
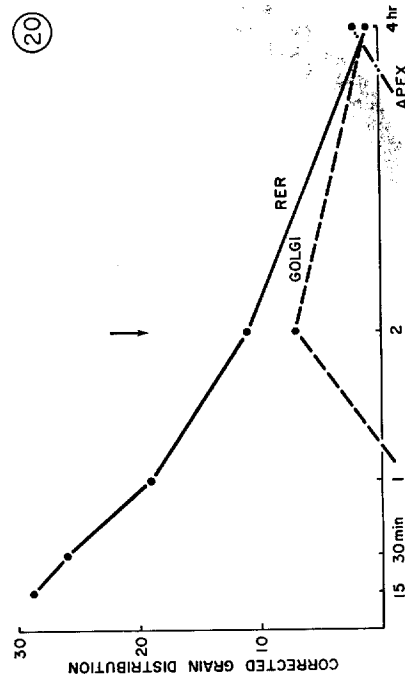
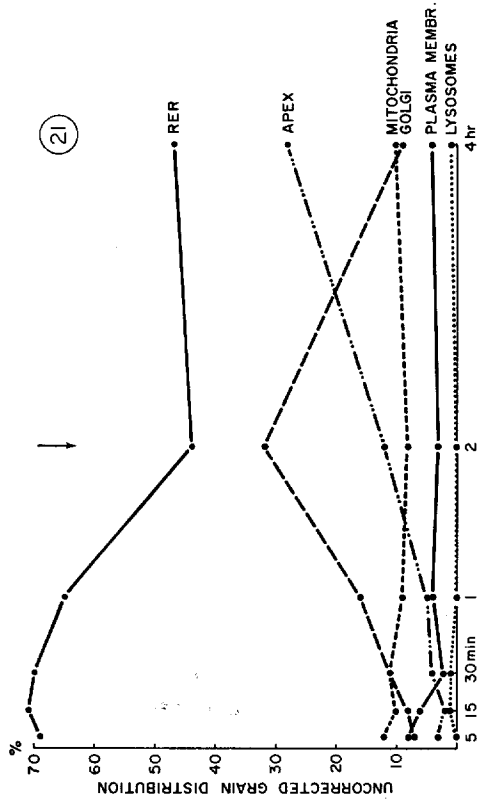
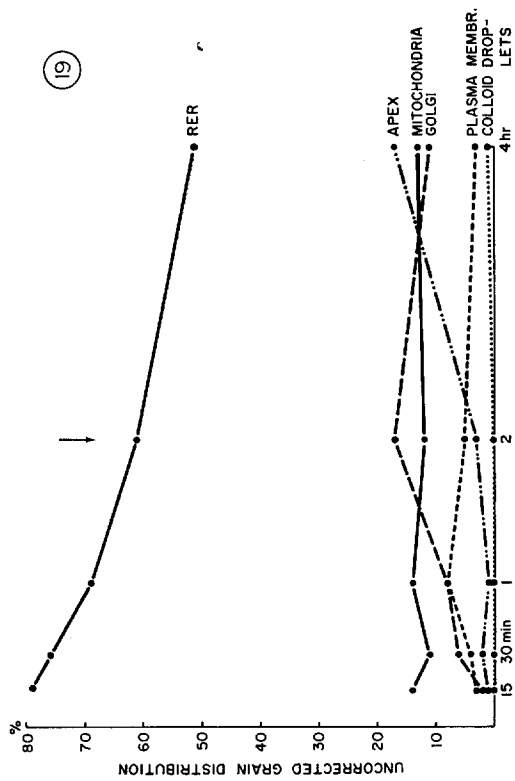
FIGURES 16–18 Electron microscope radioautographs of thyroid follicular cells from rat thyroid lobes pulse-labeled for 5 min in medium containing mannose-<sup>3</sup>H and subsequently incubated for different periods in medium containing unlabeled mannose. Figs. 16 and 18 are of preparations stained with uranyl acetate; Fig. 17, with lead citrate.

FIGURE 16 Follicular cell incubated for 5 min. The picture shows the colloid (C) at top and, below, portions of two follicular cells separated by a terminal bar (TB) and the cell membrane which is not clearly visible (CM). Except for a few mitochondria (M), the cells only show cisternae of rough endoplasmic reticulum (rER) lined with ribosomes. Four silver grains are located over the rough ER.  $\times 22,000$ .

FIGURE 17 Follicular cell incubated for 15 min, showing rough ER with dilated cisternae. The grains present are over the rough ER. By placing “95% probability” circles around the silver grains as indicated in the Methods section, it was found that the radioactive source of at least two of the grains (arrows) lies within the cisternae.  $\times 20,000$ .

FIGURE 18 Follicular cell after 2 hr of incubation. This cell shows the colloid (C) at top, the nucleus (N) at left, two dense bodies presumed to be lysosomes (L), and a prominent Golgi apparatus (G). The majority of grains are now located over the Golgi apparatus, with one grain situated beneath the cell surface adjacent to several apical vesicles (AV).  $\times 24,000$ .





FIGURES 19-22 Graphs showing the results of quantitation of electron microscope radioautographs at various time intervals after incubation of rat thyroid lobes in medium containing *mannose-3H*. The results of the first two experiments outlined in Table I are given in these graphs. Experiment 1 is presented at left in Figs. 19 and 20; and experiment 2, at right in Figs. 21 and 22. The uncorrected grain distributions are given at top (Figs. 19, 21) and the corrected ones below (Figs. 20, 22). In each one of the four graphs, an arrow indicates the approximate time at which label first enters the colloid, as shown by light microscopy.

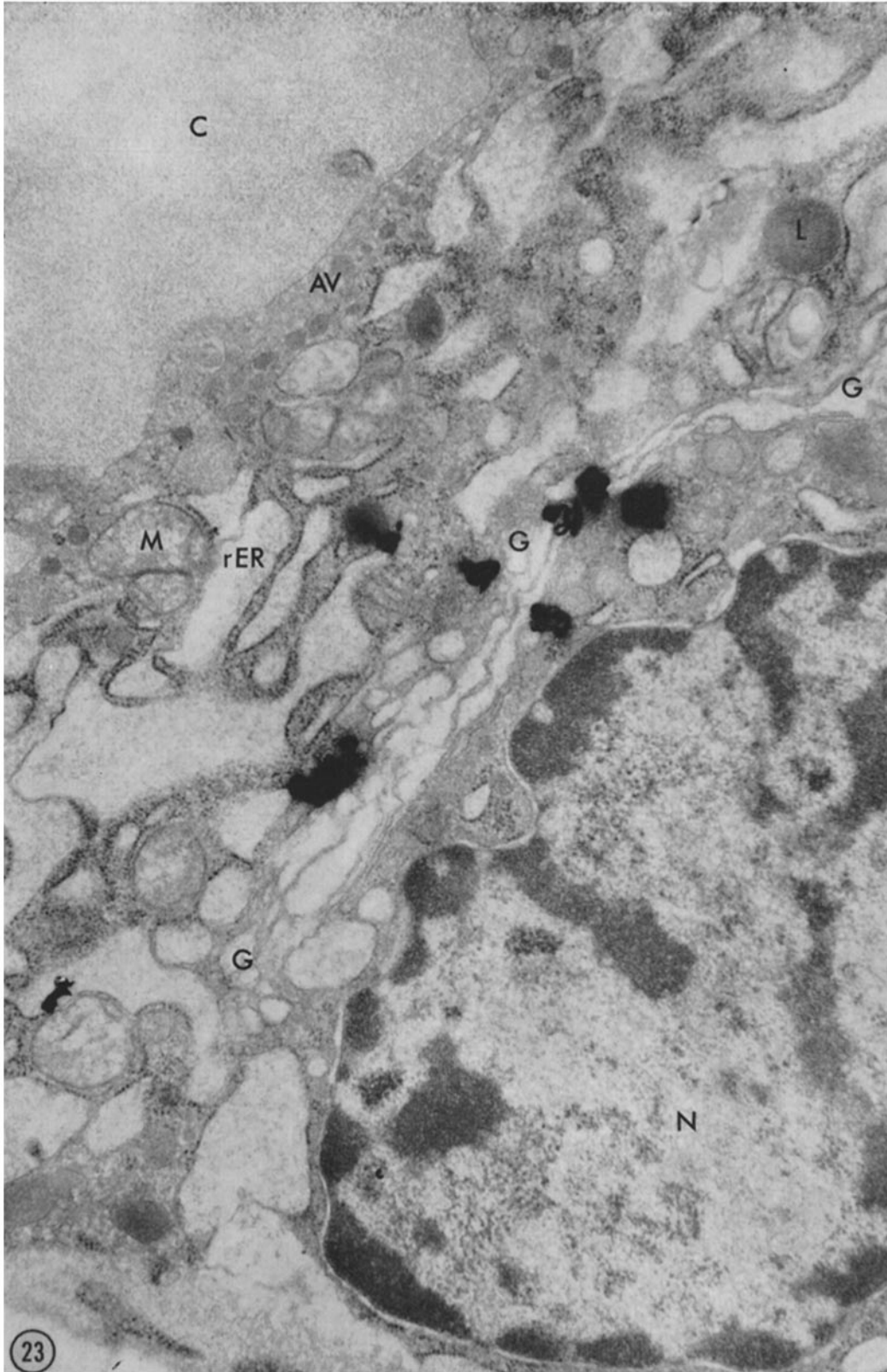


FIGURE 23 Electron microscope radioautograph of a rat thyroid follicular cell 5 min after incubation in medium containing *galactose-<sup>3</sup>H*. Stained with uranyl acetate. Lettering as in Figs. 16 and 18. Almost all the grains are located over the Golgi apparatus at this time.  $\times 31,000$ .

loss of radioactivity from the cell into the colloid. And biochemical methods showed that the specific activity of total thyroid proteins labeled with mannose- $^{14}\text{C}$  reaches a constant specific activity within the first 20–30 min of chase (25). It may be concluded, therefore, that the total amount of label within the cells remained constant between 30 min and 2 hr of chase. Therefore, any change of label within cells must be due to transfer from one organelle to another, that is, here, to transfer from rough ER to Golgi apparatus.

The time of appearance of significant grain concentration over the Golgi apparatus and apex varied in the two experiments, presumably because the timing of the events varied from follicle to follicle. For example, although colloid reactions after incubation in mannose- $^3\text{H}$  were seen in some follicles at 2 hr, they were absent in others by 4 or even 6 hr. Consequently, the times given are averages for processes continuing over fairly long periods.

In many follicles, by 2 hr, label began to leave the cell and enter the colloid. Within the cell, between 2 and 4 hr, both experiments showed a decrease in radioactivity in the Golgi apparatus and an increase in radioactivity in the apex (Figs. 20, 22). Hence, there appeared to be a transfer of label from the Golgi apparatus to the apex, before release to the colloid.

The migratory pathway described for mannose- $^3\text{H}$  label, that is, rough ER - Golgi - apex - colloid, corresponds to the pathway of the polypeptide chain of thyroglobulin, as shown by labeling with leucine- $^3\text{H}$  (12). It was concluded that the mannose label migrated with the polypeptide chains and, therefore, was attached to them.

In the case of *galactose- $^3\text{H}$* , the uncorrected grain distribution showed an initially high proportion of grains in the Golgi apparatus, which decreased with time. In addition, a large proportion also appeared over the rough ER, but remained fairly constant there throughout the experiment. Since, during that time, thyroglobulin precursors should have moved out, the persisting reaction of the rough ER did not seem to be related to thyroglobulin formation. Part of this reaction might be due to labeling of some sedentary intracellular material which, as pointed out in the Methods section, would go undetected. However, the major part of it was undoubtedly due to spurious reactions resulting from the disperse nature of the rough ER in the cytoplasm; and indeed, the corrected grain distribution, which takes into account this feature of rough ER, showed its reactions to be below the level of significance. On the other hand, Golgi reactions were significant. The Golgi apparatus, therefore, is the site of incorporation of galactose into thyroglobulin precursors. (The possibility that some galactose may also be incorporated into the rough ER cannot be ruled out.)

Beyond the 5- and 10-min intervals, the Golgi reaction decreased rapidly, while during the same period the apex reaction increased. Until 30 min, there was no loss of label to the colloid nor was there any fluctuation of the reaction in other structures; and, therefore, during this time, the label lost from the Golgi apparatus must have been transferred to the apex. A substantial part of the apex reaction was clearly attributable to apical vesicles. It was, therefore, concluded that the migration consists of a steady flow of label from

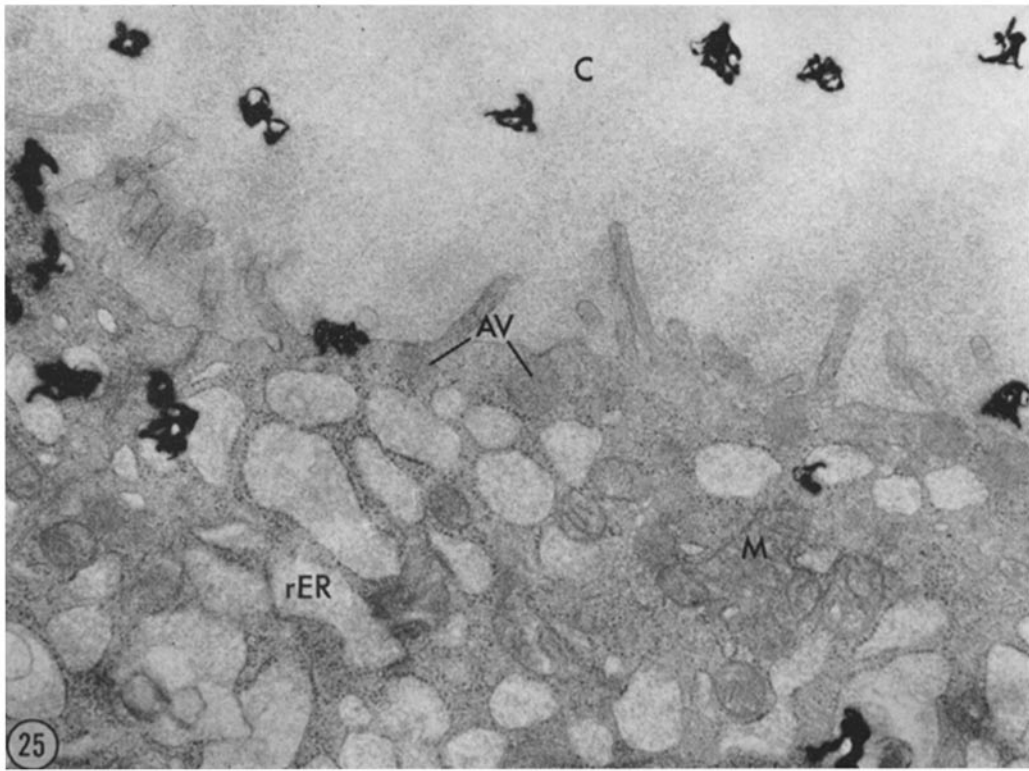
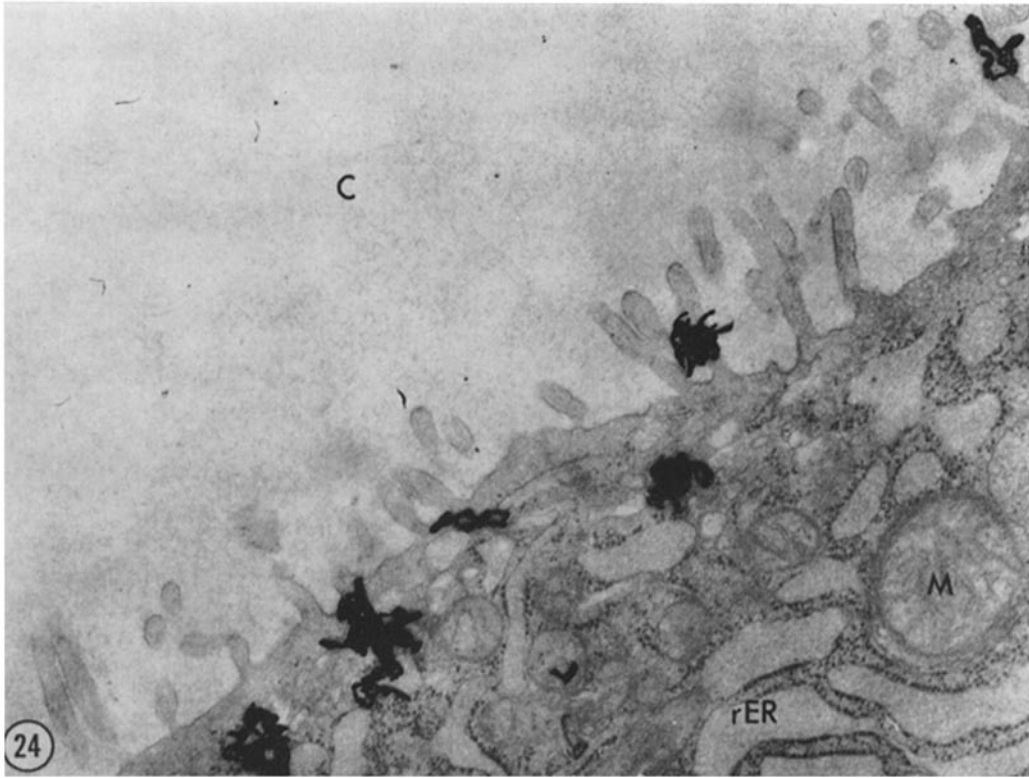
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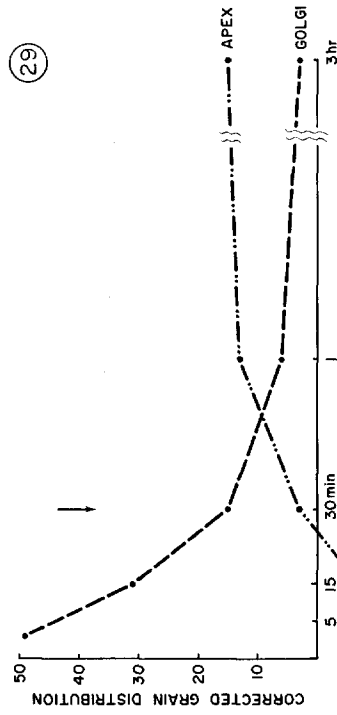
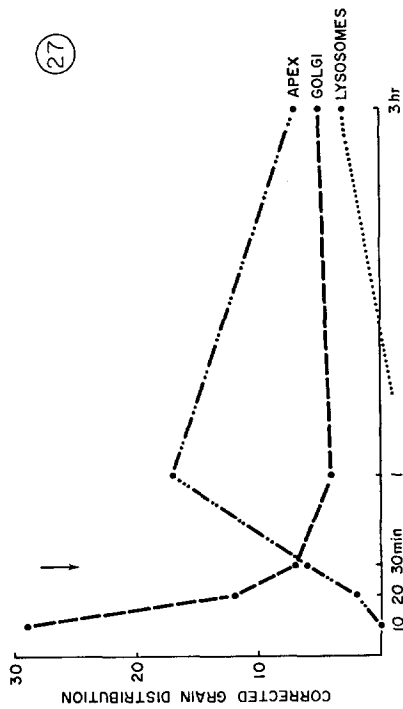
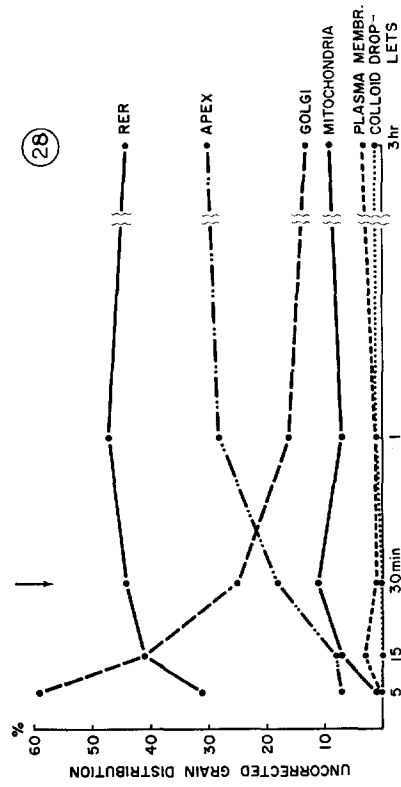
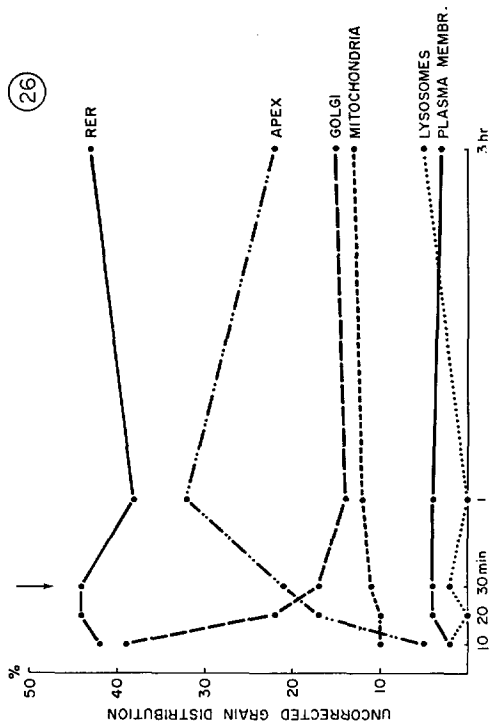
FIGURES 24–25 Electron microscope radioautographs of rat thyroid lobes after 5 min of pulse-labeling in medium containing *galactose- $^3\text{H}$*  with subsequent incubation in medium containing unlabeled galactose, to show migration of label from the site of incorporation. For Fig. 24, staining was with lead citrate, and for Fig. 25, with uranyl acetate. Lettering as in Figs. 16 and 18.

FIGURE 24 Thyroid follicular cell after 30 min of incubation. By this time the majority of grains have migrated from the Golgi apparatus and are situated beneath the apical surface of the cell.  $\times 21,000$ .

FIGURE 25 Thyroid follicular cell after 2 hr of incubation. Most of the grains are now located over the colloid, although a few remain over the cell.  $\times 23,000$ .







FIGURES 26-29 Graphs showing the results of quantitation of electron microscope radioautographs at various time intervals after incubation of rat thyroid lobes in medium containing *galactose-3H*. The results of the last two experiments outlined in Table I are given in these graphs. Experiment 3 is presented at left in Figs. 26 and 27; and experiment 4, at right in Figs. 28 and 29. The uncorrected grain distributions are at top (Figs. 26, 28) and the corrected ones below (Figs. 27, 29). In each graph, an arrow indicates the approximate time at which label first enters the colloid, as shown by light microscopy.

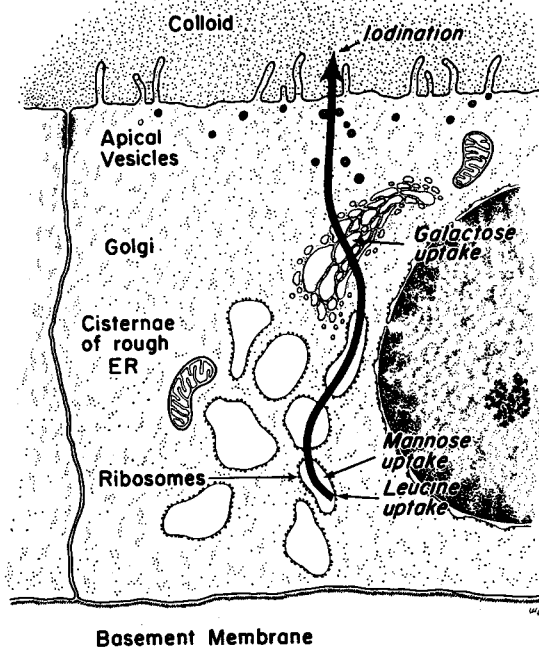


FIGURE 30 Diagram of part of a thyroid follicular cell of the rat which illustrates (main arrow) the pathway taken by thyroglobulin precursors from the time of synthesis of the polypeptide chains on the ribosomes of the rough ER, as shown by incorporation of leucine-<sup>3</sup>H label.

As demonstrated by our experiments, mannose is incorporated almost immediately afterwards, probably after the polypeptide chains have detached from the ribosome and entered the lumen of the cisternae. By 1½ hr, most of the precursors have migrated from the rough ER to the Golgi apparatus, where galactose is incorporated. Subsequently, there is a rapid transfer from the Golgi apparatus to apical vesicles and thence to the apical cell surface. Finally, iodination takes place after thyroglobulin is secreted into the colloid.

the Golgi apparatus via the apical vesicles towards the cell surface, where it enters the colloid.<sup>3</sup>

<sup>3</sup> The corrected grain distribution, as outlined in the Methods section, was obtained by calculating the percentage of grains over a given structure and subtracting from it the percentage of random hits over this structure, that is:

$$\frac{100n}{N} - \frac{100a}{A}$$

where  $n$  is the number of points recorded over the structure,  $N$  the total number of grains recorded over the cytoplasm,  $a$  the number of random hits

### General Conclusions

The radioautographic studies of Nadler et al. (12) using leucine-<sup>3</sup>H established the intracellular pathway of migration followed by polypeptide precursors of thyroglobulin from the site of their synthesis in the rough ER to the site of their secretion, the colloid. The present work demonstrates, first, that it is during this migration that the thyroglobulin polypeptides acquire carbohydrate side-chains, and secondly, that galactose and mannose are added at different cell sites in a sequence

over the structure, and  $A$  the total number of random hits over the cytoplasm. Additional information regarding concentration of the label may also be derived by calculating the ratio of the two percentages:

$$\frac{n/N}{a/A}$$

This formula may be expressed

$$\frac{n/a}{N/A}$$

which is the ratio of the concentration of grains for a given structure over the concentration of grains for the whole cytoplasm. Such ratios calculated for the mannose-<sup>3</sup>H experiments (Fig. 31) indicate that the label became highly concentrated as it was transferred from the rough ER to the Golgi apparatus and that this transfer was *continuous* from the earliest time interval. After 2 hr, as label appeared in the colloid (as shown by light microscopy), the concentration in the Golgi apparatus decreased. A priori the label present in the Golgi apparatus at 1-2 hr could have entered the colloid either directly or by transfer through the "apex," that is, through the apical vesicles. The results indicate that, in spite of the loss of label to the colloid, until 4 hr there is an apparent increase in concentration in the apex. This is taken to mean that the label associated with mannose-<sup>3</sup>H entered the colloid via the apical vesicles.

This conclusion was confirmed by expressing the results of the galactose-<sup>3</sup>H experiments in the same manner (Fig. 32). The concentration in the Golgi apparatus decreased precipitously between 5 and 20 min, while the concentration in the "apex" increased rapidly. Since this occurred before the time when label is transferred to the colloid, the label from the Golgi apparatus could only have been transferred to the "apex." It is concluded that the apical vesicles served as intermediaries for the transport of the label from Golgi apparatus to colloid.

related to their relative position in the carbohydrate side-chains (Fig. 30). Mannose, which is close to the polypeptides of thyroglobulin, is incorporated in the rough ER, whereas galactose, which is located next to the terminal sialic acid or fucose, is incorporated in the Golgi apparatus. Immediately after the polypeptides of thyroglobulin are synthesized on the polysomes of the rough ER, carbohydrate addition begins and mannose is taken up, probably as soon as the polypeptides are released into the cisternae of the rough ER. Carbohydrate addition may proceed as the precursors pass through the cisternae on their way to the Golgi apparatus, and is probably completed in the Golgi apparatus itself.

Eylar (40) has recently pointed out that most glycoproteins are extracellular. That the Golgi apparatus plays an important role in secretion of extracellular substances has been documented previously (41), and that it takes part in glycoprotein synthesis has been shown with glucose- $^3\text{H}$  in mucus-

secreting cells (42, 43) and with galactose- $^3\text{H}$  in columnar cells of intestine (44), hepatocytes (45), ameloblasts (46), and other cells (47). The Golgi apparatus is also involved in mucopolysaccharide synthesis as shown by uptake of sulfate- $^{35}\text{S}$  in cartilage cells (48) and of glucosamine- $^3\text{H}$  in synovial cells (49). The Golgi apparatus also plays a role in polysaccharide synthesis as shown with glucose- $^3\text{H}$  in plant cells (50). On the other hand, the present work is the first radioautographic demonstration that the rough ER is the site of mannose incorporation, although some biochemical results were suggestive of this possibility (39).

With the use of cell fractionation techniques in liver (51, 52) and in intestinal mucosa (53), it has been observed that glucosamine which occurs in several positions in the carbohydrate side-chains of glycoproteins may be incorporated in both the rough microsomes (derived from the rough ER) and in the smooth microsomes (including the smooth ER and the Golgi apparatus). It was then

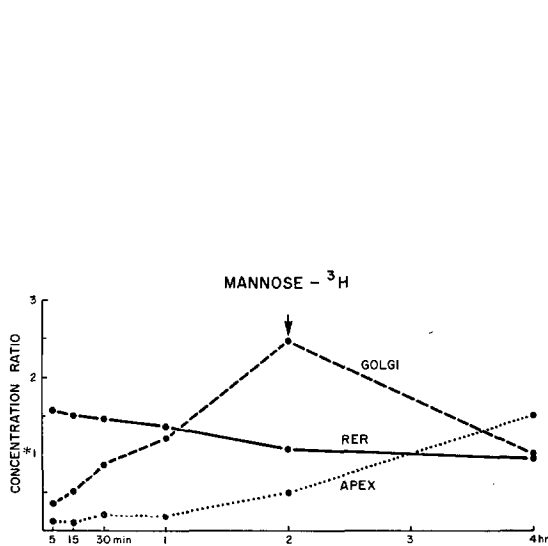


FIGURE 31

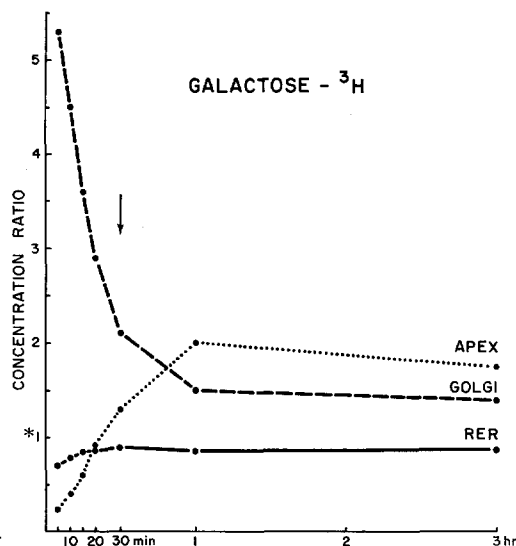


FIGURE 32

FIGURES 31-32 Graphs presenting the results of grain counts in electron micrographs as relative concentration of label (ratios of uncorrected grain counts for a structure over the relative area occupied by the structure). By expressing the data in this way, it is possible to visualize changes in concentration as well as the rate of transfer of label from one organelle to another. Arrows indicate the time of the first loss of label into the colloid as determined in the light microscope. An asterisk marks the level above which the concentration of label over a particular structure becomes significant.

FIGURE 31 Combined data from experiments 1 and 2.

FIGURE 32 Combined data from experiments 3 and 4.

suggested that glucosamine may be incorporated into glycoproteins at several subcellular sites. The present work demonstrates with radioautography that different sugars may be added in different cell organelles, namely in the rough ER and in the Golgi apparatus, and that glycoproteins such as thyroglobulin are formed by the step-wise addition of carbohydrate as the polypeptide chains migrate

through the channels of the rough ER and through the Golgi apparatus.

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