A BIOCHEMICAL AND RADIOAUTOGRAPHIC ANALYSIS OF PROTEIN SECRETION BY THYROID LOBES INCUBATED IN VITRO

LYNETTE FEENEY and STEVEN L. WISSIG

From the Department of Anatomy, School of Medicine, University of California, San Francisco, California 94122. Dr. Feeney's present address is the Department of Ophthalmology, School of Medicine, University of Oregon, Portland, Oregon 97201.

ABSTRACT

In this study we analyzed several aspects of protein secretion by thyroid follicular cells. The study was carried out on intact thyroid lobes obtained from newborn rats and incubated in vitro. The fate of leucine-^{3}H incorporated into protein within follicular cells of untreated and thyrotropic hormone (TSH)-treated lobes was traced by quantitative electron microscope radioautography. Our findings indicate that protein synthesized by the rough-surfaced endoplasmic reticulum during a pulse exposure to leucine-^{3}H is released relatively slowly by this organelle. Approximately 1 hr after onset of the pulse, a peak of radioactive protein appears in the Golgi region. The significance of this peak is not clear. Newly synthesized secretory protein passes through the apex of follicular cells without being concentrated or temporarily stored there in the form of large secretory droplets. Passage probably takes place via small vesicles which are intermingled among diverse small vesicles at the apex of the cells as well as in the Golgi region. Exposure of the lobes to TSH in the incubation medium for 45 or 90 min does not stimulate incorporation of leucine-^{3}H into protein. Acute stimulation with TSH does, however, modify the movement of secretory protein within the exocrine secretory apparatus of the follicular cell. It accelerates the arrival of the protein at the apex of follicular cells, and it accelerates the release of the protein into the follicular lumen.

INTRODUCTION

The sequence of steps by which the thyroid gland synthesizes thyroglobulin has been analyzed by fractionation procedures utilizing sucrose gradients (Seed and Goldberg, 1963, 1965; Lissitzsky et al., 1964; Vecchio et al., 1966; Thomson and Goldberg, 1968; Herscovics, 1969). Amino acid is initially incorporated into 5-8S and 12S precursor subunits of thyroglobulin. These subunits are then assembled into a 16-18S molecule which is an "immature," uniodinated form of thyroglobulin. After being iodinated, the 16-18S molecule undergoes a change in conformation which transforms it into "mature" thyroglobulin having a sedimentation coefficient of 19S (Seed and Goldberg, 1965). Thyroglobulin is a glycoprotein containing approximately 10% carbohydrate (Ujejski and Glegg, 1955; Spiro and Spiro, 1965), and the addition of carbohydrate moieties to the polypeptide chains of the molecule has also been analyzed. Particularly pertinent to the present study is the observation that mannose is incorporated at an early stage in biosynthesis, i.e., at the same stage in which amino acids are incorporated into the chains (Lissitzsky et al., 1964; Herscovics, 1969). Other carbohydrate moieties, such as galactose and fucose, are added at later stages of biosynthesis when the polypeptide

chains have already been formed and the subunit precursors of thyroglobulin are undergoing the final stages of aggregation into uniodinated 16-18S thyroglobulin (Herscovics, 1969, 1970).

By light microscope radioautography, Leblond et al. (1957) and Nadler et al. (1960) observed that, 4 hr after administration of labeled amino acids to rats and mice, there was a large amount of radioactivity in follicular colloid and a moderate amount in follicular cells. Since colloid is composed chiefly of thyroglobulin and its precursors (Gersh and Caspersson, 1940; Gersh and Baker, 1943; Smeds, 1970), these results suggested that the exocrine secretory apparatus of follicular cells had secreted substantial amounts of protein into the follicular lumen within this period. In subsequent light and electron microscope radioautographic studies, Nadler et al. (1964) examined the incorporation of leucine-³H by thyroid tissue in vivo and Whur et al. (1969) examined the incorporation of leucine-³H, mannose-³H, and galactose-³H by thyroid tissue in vitro. The findings with leucine-³H in vivo and in vitro were similar, and they resembled, in general, the pattern for synthesis of secretory protein observed in pancreatic acinar tissue (see review by Palade, 1966). At early time periods, radioactivity was localized in the rough-surfaced endoplasmic reticulum (RER) situated principally in the base of follicular cells. At 2 hr, radioactivity reached a peak in the Golgi apparatus, and radioactivity reached a maximum over presumed apical secretory vesicles at 4 hr. Of particular interest are the observations that, in the in vivo studies, the radioactivity over the RER was maximal at 0.5 hr after the administration of labeled amino acid, and 70% of the total radioactivity was still present in the RER 0.5 hr later. By 4 hr, the concentration of grains over the RER was 40% of that present at 0.5 hr. The findings obtained with mannose-³H paralleled those obtained with leucine-³H. With galactose-³H the initial site of incorporation appeared to be the Golgi apparatus, and thereafter the radioactivity moved through the apical cytoplasm to the follicular lumen.

The effect of acute stimulation with thyrotropic hormone (TSH) upon the incorporation of amino acid into protein by the thyroid gland has been investigated in a number of studies. Raghupathy et al. (1963) observed that slices of thyroid tissue obtained from guinea pigs treated with TSH for 20 hr before sacrifice showed increased incorporation of amino acid into protein. Conversely, incorporation was depressed in thyroid slices ob-

tained from hypophysectomized guinea pigs. Tong and his group (Tong, 1965, 1967; Wilson et al., 1968) noted that isolated bovine cells showed enhanced incorporation of labeled amino acid into protein when TSH was added to their incubation medium 30 min before adding the amino acid (Tong, 1967). Similar effects of TSH have also been noted under in vivo conditions. Administration of TSH to chicks (Klitgaard et al., 1965), mice (Bradley and Wissig, 1966), and rats (Schneider and Goldberg, 1965; Cavalieri and Searle, 1967) resulted in stimulation of the incorporation of amino acid into protein by the thyroid gland in situ. This effect became detectable from 2 to 4 hr after onset of stimulation (Bradley and Wissig, 1966, Cavalieri and Searle, 1967).

The effect of acute stimulation specifically upon the synthesis of thyroglobulin by the thyroid gland has not yet been extensively examined. Cavalieri and Searle (1967) observed that, in control rats which received an introperitoneal injection of labeled amino acid, 5% of the radioactivity incorporated into protein by the thyroid gland was in the form of alkali-stable 19S thyroglobulin 3 hr later. This figure increased to 15% when the rats were given TSH 1 or 2 hr before receiving labeled amino acid. The results of this study indicate that acute stimulation with TSH accelerates the rate at which mature thyroglobulin is formed from its precursor subunits. This effect was detectable 1 hr earlier than the stimulatory effect of TSH on the incorporation of amino acid into protein (see above).

The objective of the present study was to carry out a quantitative analysis, by means of electron microscope radioautography, of the sequential movement of secretory protein through the exocrine secretory apparatus of thyroid follicular cells. Since it was already clear from pioneer studies of pancreatic acinar cells (see review by Palade, 1966) and from light and electron microscope radioautographic studies of thyroid follicular cells (Nadler et al., 1960, 1964; Whur et al., 1969) that the RER is the cytoplasmic organelle in which amino acids are initially incorporated into the polypeptides of secretory protein, we focused our attention principally on subsequent steps in the secretory process. We sought to examine in detail the chronology of the passage of newly synthesized protein in sequence through the RER, Golgi apparatus, apical cytoplasm, and then into the follicular lumen. An additional portion of the study was concerned with the effect of acute stimulation with TSH on the

secretion of protein by the thyroid gland. Specifically, we examined whether the trophic hormone stimulated incorporation of amino acid into protein and movement of newly synthesized protein through the secretory apparatus. For these studies we used intact thyroid lobes removed from newborn rats and incubated under in vitro conditions. The suitability of these lobes for this type of study has already been reported (Feeney and Wissig, 1967, 1971). They do not appear to deteriorate significantly either morphologically or functionally during periods of incubation in vitro of as long as 4 hr, and they are capable of giving certain characteristic responses in vitro to stimulation with TSH. Previous workers have used thyroid lobes from adult rats for this type of study because the lobes withstand incubation in vitro better than do slices of thyroid tissue (Mack, 1966; Shimoda and Greer, 1964). However, despite the fact that small molecules such as amino acids penetrate readily to their interior, lobes from adult rats have the disadvantage that only their peripheral two or three rows of follicles do not undergo deterioration. Even among these follicles, there is evidence of asynchrony of function (Whur, et al., 1969).[1] When migration of newly synthesized secretory protein is to be traced chronologically through the secretory apparatus of follicular cells, maximal synchronization of function among individual follicles is highly desirable.

MATERIALS AND METHODS

Protein Synthesis

32 thyroid lobes were excised from newborn rats and preincubated for 45 min in sterile NCTC-109 medium containing 8 mg/100 ml bovine serum albumin (basic medium). The medium was gassed with 95% oxygen, 5% carbon dioxide before use, and all incubations were carried out at 37°C in a metabolic shaker. After preincubation, a group of eight lobes was transferred first to 2.5 ml basic medium which contained 0.05

---

[1] In their radioautographic study of the incorporation in vitro of tritiated precursors of thyroglobulin by thyroid lobes from adult rats, Whur et al. (1969) reported that, although the localization of radioactivity was similar over all cells of an individual follicle, the localization varied from one follicle to another. For example, newly synthesized radioactive protein appeared in the lumens of some follicles 2 hr after the lobes were exposed to a brief pulse of leucine-[3]H. In the majority of follicles, however, radioactive protein had not yet been secreted into the follicular lumen by 4 hr after the pulse.

units/ml TSH[2] for 30 min and then to similar medium which contained 40 μCi/ml 4,5-L-leucine-[3]H (SA 35.1 Ci/mmole)(Nuclear-Chicago Corp., Des Plaines, Ill.) but which was otherwise leucine-free[3] (pulse medium) for 15 min. The lobes were then transferred to basic medium containing 4 mM unlabeled leucine (chase medium) for 15 min. A second group of eight lobes was treated similarly except that their initial incubation with TSH lasted 75 min and they were then pulse labeled with 200 μCi/ml 4,5-L-leucine-[3]H. A control group of eight lobes was run with each TSH-treated group. None of the incubation media of the control groups contained TSH.

Lobes of the TSH-treated and control groups were homogenized in 2 ml ice-cold 0.05 M carrier leucine solution. Multiple samples of each homogenate were dried and solubilized in Nuclear-Chicago Solubilizer (NCS) reagent by heating at 60°C for 1 hr. Scintillation fluid was added, and the total radioactivity was determined with a Packard Tri-Carb counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Other samples of each homogenate were placed in centrifuge tubes to which 3 mg bovine serum albumin was added, and protein was precipitated either by a standard tricholoracetic acid (TCA) procedure (Allen and Schweet, 1962) or by a revised TCA procedure.[4] The precipitated protein was solubilized, and multiple

---

[2] Thytropar, bovine thyroid-stimulating hormone, from Armour Pharmaceutical Company, Chicago, Ill.

[3] Leucine-free NCTC-109 from the Colorado Serum Co., Denver, Colorado.

[4] In a preceding study (Feeney and Wissig, 1971), we observed that there was substantially less radioactive protein in thyroid lobes labeled with ^{125}I when protein was precipitated according to a slightly modified Allen and Schweet (1962) procedure versus when the protein was precipitated with a histologic fixative. In the modified Allen and Schweet procedure, protein was precipitated with TCA at a final concentration of 7% at 0°C. By measuring the radioactivity in each of the subsequent wash solutions, we determined that substantial amounts of the ^{125}I-labeled protein which was initially precipitated with TCA were dissolved by the ethanol wash solution. Thereafter we found that solubilization of the precipitated protein by ethanol was minimized when the initial precipitation was carried out as follows: TCA was added to the homogenate to yield a final concentration of 15%, and the mixture was placed in a boiling water bath for 5 min. Similar instances of the solubilization in ethanol of TCA-precipitated bovine serum albumin (Levine, 1954) and proteins of liver fractions (Munro and Downie, 1964; Kaltenbach, 1969) have been reported. In two of these instances (Levine, 1954; Kaltenbach, 1969), the solubilization was also averted by heating during the initial TCA precipitation.

samples were counted by liquid scintillation as above to determine protein-bound radioactivity. Quenching was determined with an internal standard. With the remainder of the homogenate, total protein was determined by the method of Lowry et al. (1951). Porcine thyroglobulin was used as the standard.

Radioautography

TSH-treated and control groups of thyroid lobes from newborn rats were subjected to a procedure for pulse-chase labeling of protein similar to that described above. The same preincubation, pulse, and chase media were used. TSH-treated lobes were preincubated in basic medium for 45 min and were transferred to basic medium containing 0.05 units/ml TSH for 60 min. They were then placed in pulse medium containing 200 μCi/ml 4,5-L-leucine-^{3}H and 0.05 units/ml TSH for 15 min. The lobes were transferred to chase medium without TSH for various periods of time ranging from 15 to 195 min. At the end of the chase, they were placed in fixative. The control lobes were treated similarly except that they were preincubated for 105 min and were placed in pulse medium without TSH for 15 min.

The lobes were fixed with a modification of Karnovsky's (1965) glutaraldehyde-formaldehyde mixture.[5] The modified fixative contained 2% glutaraldehyde, 1% paraformaldehyde, 0.067 M sodium cacodylate buffer, pH 7.4, 0.007 M KCl, 0.02% $CaCl_2$, and 1% sucrose. The lobes were placed in this fixative overnight and were then transferred to 2% OsO_4 buffered at pH 7.5 with phosphate or veronal-acetate buffer for 2 hr. They were stained en bloc with uranyl acetate according to the method of Kellenberger as modified by Farquhar and Palade (1965). They were

[5] Several reports have appeared in the literature stating that glutaraldehyde used as a fixative can cause nonspecific binding of free labeled amino acid to protein (Droz, 1967; Hodson and Marshall, 1967; Peters and Ashley, 1967; Whur et al., 1969). This could be a pitfall for radioautographic studies, but, in most instances, it actually is not. In the first place, the amount of amino acid that is nonspecifically bound is small in proportion to the amount that is being incorporated by a functioning tissue (Hodson and Marshall, 1967). The amount nonspecifically bound is further reduced in relative significance when the labeled amino acid is chased from the tissue before fixation (Droz, 1967; Peters and Ashley, 1967). Peters and Ashley (1967) conclude, therefore, that nonspecific binding of labeled amino acid to protein by fixation is a hazard only when duration of exposure of the tissue to amino acid is very brief and when there is a significant amount of free amino acid present in the tissue at the time it is placed in fixative. Neither of these conditions applied in the present study.

dehydrated in ice-cold acetone and embedded in Epon (Luft, 1961).

Thin sections of lobes were cut with a Sorvall MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.) equipped with a diamond knife. The sections were mounted on copper grids coated with a parlodion film and were stained sequentially with uranyl and lead salts (Venable and Coggeshall, 1965). We verified, from radioautographs of thyroid tissue which had not been exposed to leucine-^{3}H, that staining with uranyl acetate did not itself cause an radioautographic reaction. The grids were coated with a layer of carbon by vacuum evaporation and were attached in pairs (TSH-treated and a corresponding control specimen) to slides with tape having adhesive on both sides. The slides had been "subbed" with a 0.5% gelatin, 0.01% chromium potassium sulfate mixture. The slides were arranged in sets, each set containing grids from all of the chase intervals examined in this study.

Ilford L-4 emulsion (Ilford Ltd., Ilford, England) (1 part emulsion: 1.5 parts water) was liquefied in a water bath at 45°C, and a film of this emulsion was applied to the grids by the method of Caro and van Tubergen (1962). Tests were made at the time of filming to verify whether the thickness of the film of emulsion was satisfactory and whether the level of background fog was minimal. During exposure the grids were stored with Drierite (W. A. Hammond Drierite Co., Xenia, Ohio) in lighttight boxes at room temperature. The times of exposure ranged from 3 wk to 3 months. The sets of slides were developed in filtered Microdol X at 20-22°C for 5 min and fixed in filtered Kodak Acid Fixer for 4–5 min. Sections used for making grain counts were photographed without previous removal of the gelatin of the emulsion. The gelatin was removed from other sections by floating them on 0.05 N NaOH. We did not detect any removal of grains by the treatment with dilute NaOH.

The concentration of grains over the nucleus, RER, Golgi apparatus, mitochondria, a zone of cytoplasm 1 μ wide along the apical border, and the cytoplasmic matrix[6] of follicular cells and over the follicular colloid was determined from each radioautograph. To do this, areas of follicular cells adjacent to the follicular lumen were photographed at × 4000 or × 10000 magnification with a Siemens Elmiskop I, and each micrograph was enlarged × 2.8 during printing. The grains over each of the structures listed were counted in each micrograph. Individual grains were assigned to a particular structure when the structure was located within a circle of probability of 75% surrounding each grain (Bachmann and Salpeter, 1965). The

[6] Structured and unstructured components of follicular cells not included in the other listed categories were arbitrarily assigned to the cytoplasmic matrix.

circle had a radius equivalent to 1250 A, and the center of the circle was placed at the midpoint of a straight line linking the two ends of the grain. When more than one structure occupied the circle of probability, fractions of a grain were assigned each structure based on the fraction of the total area of the circle occupied by the structure. Each print was then perforated with 108 regularly spaced pinholes, and the structures perforated by the holes were counted. From the pinhole counts we calculated the per cent of the total area of each micrograph occupied by each structure. In order to correct for photographic background, developed grains over measured areas in grid openings without tissue were counted and expressed as grains per unit area. The background was subtracted from the grain counts of each structure on the basis of the area occupied by the structure. Photographic background was less than 1 grain/15.4 μ^2. The corrected grain counts for each micrograph were used to calculate the percentage of the total grains over each structure.

The data from area measurements and grain counts were combined and expressed as:

Relative grain concentration

$$= \frac{\% \text{ of total grains over a structure}}{\% \text{ of total area occupied by a structure}}$$

The relative grain concentration over the various structures was determined at 30, 60, 120, and 210 min after the thyroid lobes were exposed in vitro to a pulse of labeled amino acid. The values for relative grain concentration obtained from individual micrographs for each chase interval were pooled, and mean and standard deviations were calculated. By this procedure we essentially followed changes in *concentration* of radioactive protein in the structures at intervals after the pulse.

OBSERVATIONS

Table I summarizes the data from the portion of this study designed to assess the effect of TSH on the capability of thyroid lobes incubated in vitro to carry out the synthesis of protein. The data from two separate experiments are shown. They indicate, first of all, that the lobes are capable of taking up amino acid from the incubation medium and incorporating it into protein. However, the proportion of the total radioactivity in the lobes, which appears in the TCA precipitate and therefore is assumed to be incorporated into protein, differs in the two experiments; it is 21% in the TSH-treated and control groups of the first experiment whereas it is 100% and 83%, respectively, in the corresponding groups of the second experiment. The explanation for this lies in the fact that the TCA precipitation procedure used in the first experiment allowed a considerable amount of the precipitated protein to be extracted in the succeeding ethanol wash (see Materials and Methods). The amount extracted approached 80%. Other workers have reported similar results with similar TCA precipitation procedures. For example, depending upon the particular TCA procedure employed, ethanol and other lipid solvents can extract 35–65% of precipitated liver proteins (Munro and Downie, 1964; Kaltenbach, 1969). Despite the fact that a large portion of the TCA-precipitated radioactivity was extracted by ethanol in the first experiment, we assume that the amount extracted was proportionately the same in the control and TSH-treated groups, and therefore the data are still valid for a comparison of the effect of TSH. In our second experiment, with the use of a higher

TABLE I

Leucine-^3H Incorporation into Protein by Incubated Thyroid Lobes

	Total incorporation	TCA precipitate	TCA precipitate as % of total incorporation
	cpm/μg protein	*cpm/μg protein*	
TSH (45 min)*	1058 ± 87	226 ± 5‡	21
Control*	1135 ± 92	243 ± 40‡	21
TSH (90 min)§	5142 ± 870	5190 ± 1050‖	100
Control§	6152 ± 1320	5104 ± 1111‖	83

* The medium contained 40 μCi/ml 4,5-L-leucine-^3H during the final 15 min of incubation with TSH.
‡ Standard TCA procedure.
§ The medium contained 200 μCi/ml 4,5-L-leucine-^3H during the final 15 min of incubation with TSH.
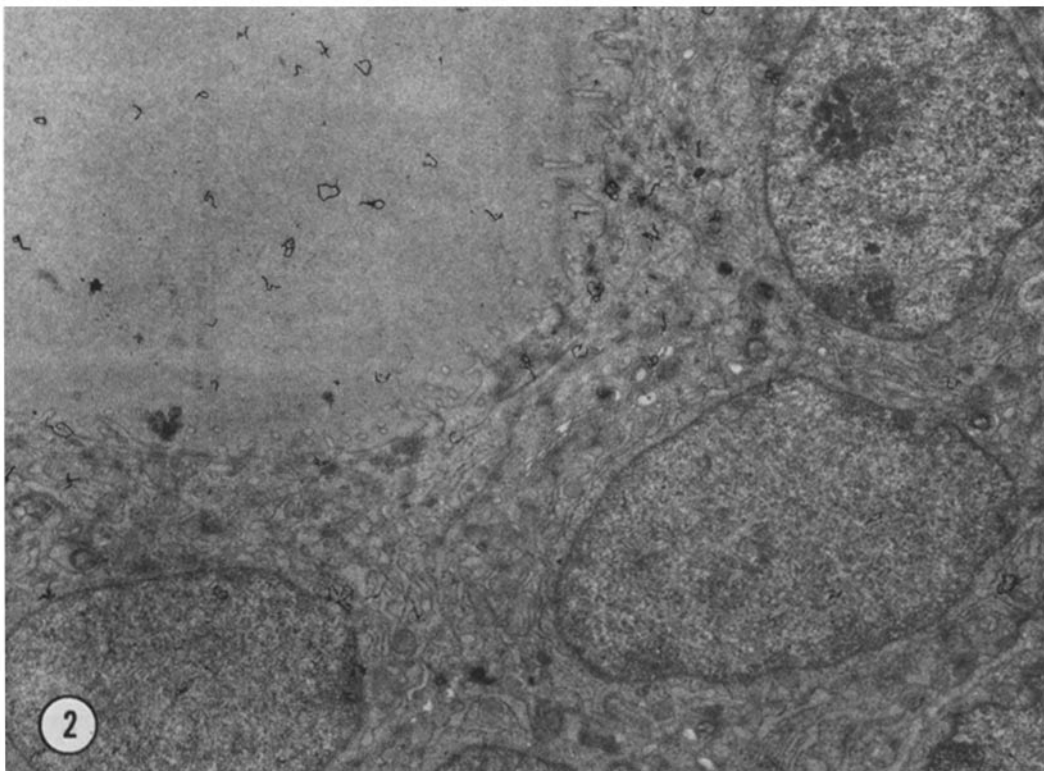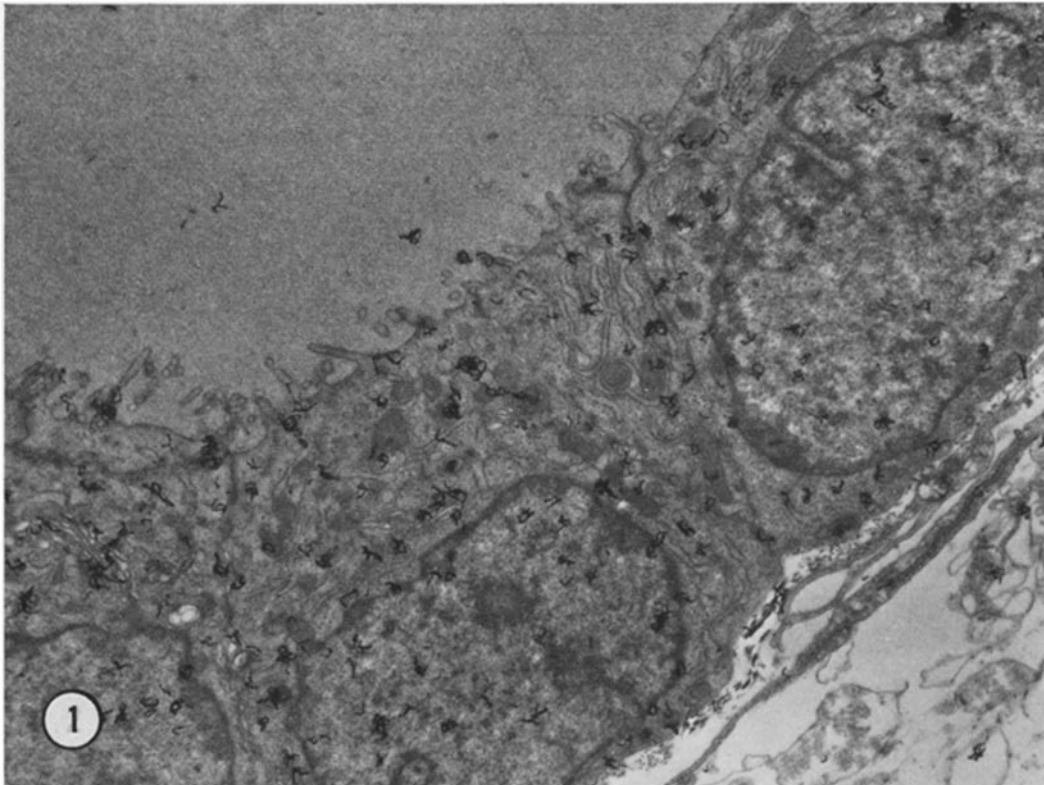‖ Revised TCA procedure.

FIGURE 1   Electron microscope radioautograph of follicular cells in a thyroid lobe incubated with TSH for 75 min. Leucine-³H was added to the medium for the last 15 min of the incubation. The lobe was then incubated in chase medium for 45 min. Therefore, this is a 60 min TSH-treated lobe. Silver grains appear over the follicular cells, and they appear to be concentrated over the Golgi apparatus. There are essentially no grains over the follicular colloid. × 8000.

FIGURE 2   Electron microscope radioautograph of follicular cells in a control lobe pulse labeled with leucine-³H for 15 min and then incubated in chase medium for 195 min. Therefore, this is a 210 min control lobe. Grains are located over both the cells and the follicular colloid. × 8000.

concentration of TCA and heating during the initial precipitation step, 83% or more of the total radioactivity in the lobes was determined to be protein-bound. This is, incidentally, a good indication that the 15-min chase used in these and the radioautographic experiments washed out nearly all unincorporated leucine-^{3}H from the lobes. When we compare the relative amount of labeled amino acid incorporated into protein by the TSH-treated and control lobes of each experiment, we again find that they do not differ. These findings indicate that, under the experimental conditions used in this study, exposure of thyroid lobes to the trophic hormone for as long as 90 min does not affect the rate at which the lobes incorporate amino acid into protein.

Representative examples of electron micrographs from the radioautographic portion of the study are shown in Figs. 1 and 2. The portion of a follicle shown in Fig. 1 is from a lobe which was stimulated with TSH, pulse-labeled with leucine-^{3}H for 15 min, and then incubated in chase medium for 45 min. The cells in this particular field do not show the characteristic response of colloid phagocytosis that follows acute stimulation with TSH. (In this study we observed that the number of radioautographic grains over colloid droplets was minimal, and we did not include these structures in our grain counts.) The large number of grains over the follicular cells is an additional indication that their capability for protein synthesis is effective during incubation in vitro. The small number of grains over the luminal colloid signifies that the bulk of the radioactivity incorporated into

protein is, after this chase interval, still localized within the follicular epithelial cells. At a glance, the grains over the cells appear to be distributed at random, although there is some suggestion that they are more concentrated over the Golgi region.

Fig. 2 shows a portion of a control lobe that was incubated in chase medium for 195 min after 15 min pulse labeling with leucine-^{3}H. This was the longest chase period used in this study. After this chase interval, an appreciable number of grains appears over the colloid, and relatively fewer grains appear over the follicular cells. This indicates that the exocrine secretory apparatus of the cells has emptied a substantial part of its radioactive product into the follicular lumen. Once again the grains over the follicular cells, fewer in number in this instance, appear to be distributed at random.

More meaningful conclusions concerning the localization of radioactive protein at various intervals after a pulse exposure to leucine-^{3}H were drawn on the basis of grain counts. These data are presented in Table II and Fig. 3. Table II shows the distribution of grains over various follicular structures at intervals after the pulse as percentages of the total number of grains. Fig. 3 shows the relative concentration of grains over the same structures at corresponding intervals.

In Table II we note that the percentage of total grains over the RER decreases during the period from 30 to 210 min after onset of the pulse-exposure to leucine-^{3}H. The decrease during this interval is of the order of 30–50% and is similar in magnitude in the TSH-treated and control groups. In the

TABLE II

Grain Distribution in Follicles of Thyroid Lobes Labeled In Vitro with Leucine-^{3}H*

| Min after onset of pulse | % of total grains‡ | | | | | | Total grains | TSH§ |
	RER	Golgi	Apical (1 μ)	Colloid	Matrix	Nucleus		
30	23	12	13	3	25	24	727	—
60	21	14	21	4	19	16	445	—
120	11	14	31	2	24	16	500	—
210	11	8	21	27	21	11	531	—
30	18	8	16	6	31	21	512	+
60	11	20	20	4	22	22	1108	+
120	10	12	24	14	32	6	627	+
210	13	4	20	25	23	16	556	+

* The lobes were pulse labeled for 15 min with 200 μCi/ml 4,5-L-leucine-^{3}H.
‡ Averages of percentages calculated from individual micrographs.
§ The treated lobes were exposed to TSH during the 15 min pulse and during the preceding 60 min incubation period.
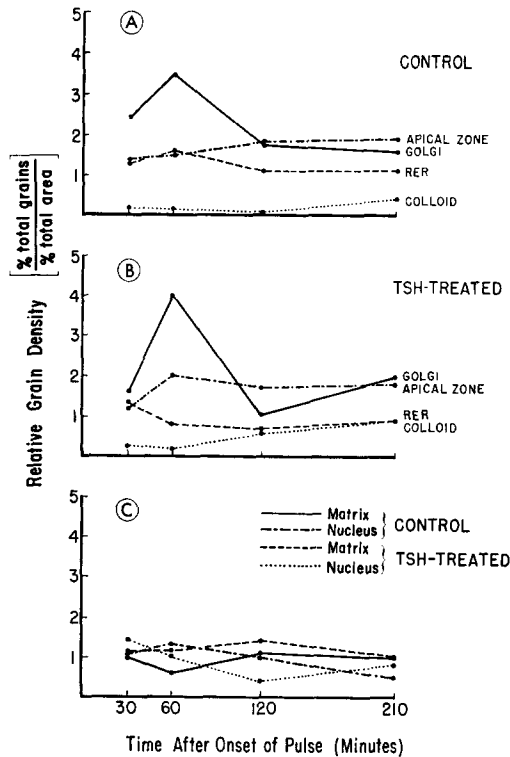
FIGURE 3 These graphs show the relative grain densities over organelles of follicular cells and over the follicular colloid at intervals after onset of a 15 min pulse exposure to leucine-³H. Data from control and TSH-treated lobes are plotted in Figs. 3 A and 3 B, respectively. Data for the nucleus and cytoplasmic matrix of both control and TSH-treated groups are plotted in Fig. 3 C. The experimental protocols and the calculations are described under Materials and Methods.

control lobes, the largest decrease in radioactivity occurs in the interval from 60 to 120 min after the onset of the pulse. A decrease of similar magnitude occurs in TSH-treated lobes except that it occurs earlier, i e., in the interval from 30 to 60 min after onset of the pulse. The earlier occurrence of the decrease in TSH-treated cells suggests that acute stimulation with the trophic hormone accelerates the transfer of newly synthesized protein out of the RER.

In both groups the percentage of grains over the Golgi complex is highest during the interval from 60 to 120 min after onset of the pulse. This suggests that a "bolus" of radioactive protein passes through the Golgi apparatus during this interval. The percentage of grains over the apical zone of the follicular cells tends to increase with time in

both groups, but the trend is not pronounced. Possible explanations for this will be discussed later. In the control lobes, the percentage of grains over the colloid is low until the 210 min interval at which time there is a sharp increase. This indicates that in these lobes newly synthesized secretory protein, presumably thyroglobulin, begins to leave the follicular cells between 120 and 210 min after onset of the pulse. In the TSH-treated lobes, there is a significant increase in the percentage of grains over the colloid at an earlier period, i.e., at 120 min after onset of the pulse. This suggests that radioactive thyroglobulin is released earlier from follicular cells acutely stimulated with TSH.

In both groups of lobes, a substantial percentage of the total grains was localized over the cytoplasmic matrix and the nucleus at all time intervals (Table II). In some instances the values of the percentages for both the cytoplasmic matrix and the nucleus fluctuated erratically at successive time intervals and did not follow a consistent pattern. This was particularly true for the grain counts over the nucleus. The erratic fluctuations probably stem from an inherent shortcoming of the counting procedure, i.e., grains over substantially different areas of these organelles were counted at each interval. This seems to be the correct explanation because, when the radioactivity in the nucleus and cytoplasmic matrix is expressed in terms of the concentration of grains over each organelle (Fig. 3 C), the concentration fluctuates little throughout the course of the experiment.

The data concerning the relative concentration of grains over various follicular structures at intervals after the pulse of leucine-³H are presented in Fig. 3. They confirm and extend the observations described above, based on total grain counts. By following the changes in concentration of grains over structures, we are essentially following changes in concentration of radioactive protein in these structures after pulse labeling with leucine-³H. These data are probably more reliable than are those related to percentage of total grain counts since they are not biased by variations in the total area of a particular structure counted from one experiment to another.

The concentration of radioactive protein in the RER shows only a slight decline during the period from 30 to 210 min after the pulse. This decline is less than that perceived on the basis of total grain counts. In these data we again detect a bolus of radioactive protein passing through the Golgi ap-

paratus of control and TSH-treated follicular cells during the interval of from 30 to 120 min after onset of the pulse. Our data indicate that this radioactivity reaches a peak of concentration at 60 min. We do not have sufficient time points within this interval to define more precisely the temporal location of the peak. From 120 to 210 min, the level of radioactivity in the Golgi apparatus remains stable.

There is substantial radioactivity in the 1μ wide zone along the apex of follicular cells in control lobes at 30 min after onset of the pulse. The concentration of grains in this zone equals that in the RER and increases slowly during the next 180 min. The gradual increase is consistent with the fact that the newly synthesized, radioactive protein must pass through this zone on its way to the follicular lumen. The relatively high concentration at the earliest intervals needs further explanation. It is probably attributable to the presence in this zone of small cisternae of the RER. They are difficult to recognize as such as they are small and sparsely coated with ribosomes (Feeney and Wissig, 1971), and they are not readily distinguishable morphologically from vesicles in this region which are presumed to be carrying protein to or from the follicular lumen (Nadler et al., 1964; Whur et al., 1969; Seljelid et al., 1970; Haddad et al., 1971). Moreover, the resolution of the radioautographic technique (Caro, 1962; Bachmann and Salpeter, 1965) is not sufficient to discern whether they or closely neighboring structures contain radioactive material. Nevertheless, we can assume that these cisternae, like cisternae of the RER elsewhere in the cell, are synthesizing radioactive protein and thereby contributing to the grain count in the apical zone at the early intervals.

In the TSH-treated lobes the concentration of radioactivity in the apical zone is also equivalent to that in the RER at the earliest interval. However, at 60 min it is sharply increased. The difference between control and TSH-treated lobes in radioactivity in the apical zone at 60 min has a P value of < 0.05. At the later time intervals, the concentration of radioactivity in the apical zone in control and TSH-treated cells was similar. These results indicate that TSH accelerates the rate at which newly synthesized protein reaches the apical zone on its way to being released into the follicular lumen.

The data on concentration of grains over the follicular lumen confirm what we noted earlier

from the data on total grain counts, i.e. in control lobes radioactive protein is first detected in the colloid at 210 min, whereas in TSH-treated lobes it is first detected at 120 min. The difference in grain concentration over the follicular lumen in control and TSH-treated lobes at 120 min after onset of the pulse has a P value of < 0.01. Therefore, acute stimulation with TSH causes follicular cells to release newly synthesized secretory protein, presumably thyroglobulin, earlier into the follicular lumen.

DISCUSSION

The radioautographic portion of the study furnishes data on the movement of newly synthesized protein through the exocrine secretory apparatus of the follicular cell of the rat thyroid. The thyroid lobes were exposed in vitro to a 15 min pulse of leucine-^{3}H, and the movement of radioactive protein was followed during the interval from 30 to 210 min after onset of the pulse. The RER is assumed to be the organelle in which the secretory protein is synthesized initially (Siekevitz and Palade, 1960; Nadler et al., 1964). The total grain counts from the control lobes indicate that, at 30 min after onset of the pulse, a substantial portion (23%) of the radioactivity in follicular cells is in the RER. Although on the basis of the total grain counts, the radioactivity in the RER appears to decline at a significant rate during the course of experiments, the rate of decline is appreciably less when the counts are corrected for area of this organelle and we, in effect, assess change in concentration of radioactive protein. Therefore, in the interval from 30 to 210 min after onset of the pulse, the RER of the follicular cell releases newly synthesized protein slowly from its cisternae.

The question may be raised whether the RER contained greater amounts of radioactive protein at the end of the pulse, and released the bulk of it during the first 15 min of the chase period. Although neither this nor earlier radioautographic studies of amino acid incorporation provide quantitative data on this early interval, cell fractionation and radioautographic studies of mannose incorporation by follicular cells indicate it is highly unlikely that this is the case. By radioautography Whur et al. (1969) localized the site of incorporation of mannose, which is incorporated into the monomeric precursors of thyroglobulin along with the amino acids, as the RER. This study also showed that, over a period extending from the end

of a 5 min pulse exposure to mannose-^{3}H to 235 min later, the relative concentration of radioactivity in the RER decreased at a uniform rate by a total of approximately 30%. The rate of decline closely parallels that observed in our study and indicates that protein newly synthesized by the RER is not released at an early interval but is retained for relatively long periods. Therefore, the turnover of protein, whether secretory or nonexportable, within the RER of thyroid follicular cells occurs slowly.

By comparison, the turnover rate of secretory protein in the RER of the pancreatic acinar cell is much more rapid. In slices of guinea pig pancreas pulse-labeled in vitro for 3 min with radioactive amino acid, Jamieson and Palade (1967a, b) showed, on the basis of cell fractionation and radioautographic studies, that the RER's content of radioactive protein falls sharply within a brief chase interval. At the end of a chase period of 7 min, approximately 50% of the radioactive protein present at the end of the pulse left this organelle. The marked difference in turnover rate of newly synthesized secretory protein in the RER of the pancreatic acinar and thyroid follicular cells may be related to differences in the ultrastructure of the RER of the two cells. There are many fewer ribosomes per unit surface area of the RER of the follicular cell (Feeney and Wissig, 1971), and the cisternae of the RER of this cell are distended with stored material (Wissig, 1960). These ultrastructural features should result in limited protein synthetic capability at the membrane of the RER and dilution intracisternally of newly synthesized protein in a large pool of stored protein. Both features would contribute toward the slow turnover of protein in the follicular cells' RER observed in this as well as previous studies.

In both the control and TSH-treated lobes, we observed a prominent peak in radioactivity in the Golgi apparatus at 60 min after onset of the pulse of leucine-^{3}H. Whur et al. (1969) observed a similar peak of radioactivity in the Golgi apparatus after follicular cells were pulse-labeled with mannose-^{3}H, although in their study the peak occurred somewhat later. This phenomenon does not seem, therefore, to be an artifact. Nevertheless, at this point it is difficult to offer an explanation for it. During the period when radioactivity was increasing sharply in the Golgi apparatus, we did not detect a corresponding sharp decrease in radioactivity in the RER. Similarly during the period

when radioactivity was decreasing sharply in the Golgi apparatus, we did not detect a correspondingly sharp increase in the succeeding component of the exocrine secretory apparatus which should be located somewhere in the apical zone of the follicular cell. Hopefully, future studies will offer some insight into the significance of the peak of radioactivity in the Golgi apparatus.

In the preceding section we noted that, in control lobes, there was a substantial amount of radioactivity in the apical zone at the earliest intervals and it increased only slowly thereafter. The radioactivity present at the outset can be attributed to the presence of cisternae of the RER in this zone. The minimal increase in radioactivity observed at later intervals, presumably when newly synthesized radioactive protein is passing from the Golgi apparatus to the follicular lumen, indicates that the passage of this material takes place at a continuous and rapid rate and that the secretory product is neither stored nor concentrated before its release. This is consistent with the fact that conventional secretory droplets have not yet been identified in the follicular cell, and those large droplets or granules which are present in the apex of the cell contain resorbed colloid or are related to lysosomes (Nadler et al., 1962; Stein and Gross, 1964; Wetzel et al., 1965; Seljelid et al., 1967 b). Undoubtedly the transport of secretory protein from the Golgi apparatus to the apical surface is performed by small vesicles (Nadler et al., 1964; Whur et al., 1969; Haddad et al., 1971). Small vesicles of several diverse types are present in this region and have been categorized on the basis of differences in density of content, size, and thickness of limiting membrane (Feeney and Wissig, 1971), their ability to incorporate tracers introduced into the follicular lumen (Seljelid, 1967 a; Seljelid et al., 1970), and their content of peroxidase (Strum and Karnovsky, 1970, 1971). As yet we do not have a foolproof means for singling out, from the clusters of vesicles near the apical border, those which are transporting thyroglobulin to the follicular lumen. It is clear that the conventional electron microscope radioautographic technique used in this study to localize ^{3}H-labeled protein has a resolution in the range of 1500–2000 A (Bachman and Salpeter, 1965) and therefore lacks this capability. Thus it is necessary to bear in mind that, although the transport of secretory protein from the Golgi apparatus to the follicular lumen does take place via small vesicles, the vesicles in

the apical region appear to be, structurally and functionally, a mixed population, and it is difficult to distinguish among them those which are transporting newly synthesized thyroglobulin.

After stimulating the thyroid lobes in vitro with TSH for up to 90 min, we did not observe an increase in the amount of labeled amino acid incorporated into protein. This is consistent with results that have been obtained in vivo. Under in vivo conditions TSH does stimulate increased incorporation of amino acid into protein by the thyroid gland, but the effect is first detectable after a latency period of 2–4 hr (Bradley and Wissig, 1966; Cavalieri and Searle, 1967). It is interesting to note that, with isolated bovine thyroid cells, Tong (1967) detected that TSH stimulated incorporation of amino acid into protein after only 30 min. The shorter latency period with this type of in vitro preparation may be the result of increased sensitivity of isolated cells or may reflect a species difference.

We observed that TSH has a prompt effect on other aspects of protein secretion by thyroid follicular cells, i.e., it accelerated the rates at which newly synthesized secretory protein arrived at the apical zone of follicular cells and was thereafter released into the follicular lumen. Therefore, TSH accelerated the rate at which newly synthesized thyroglobulin passed through the terminal component of the exocrine secretory apparatus. These findings may be related to that of Cavalieri and Searle (1967) who reported that TSH, after a latency period of 1 hr, enhanced the rate at which 19S thyroglobulin is formed from its subunit precursors. Although we do not yet know where within the exocrine secretory apparatus of the follicular cell thyroglobulin is assembled from its precursor subunits, it is possible that the two processes, i. e., the assembly of the molecule and its movement through successive compartments of the secretory apparatus, are interdependent.

Acute stimulation with TSH stimulates phagocytosis of colloid by follicular cells (Nadler et al., 1962), and the large colloid droplets formed fuse promptly with preexisting lysosomes which migrate to meet them from the base of the follicular cells (Wetzel et al., 1965; Seljelid, 1967 b). Coincident with these morphologic changes is the enhanced release of thyroid hormone from the thyroid gland indicating increased hydrolysis of the resorbed colloid (Tonoue et al., 1970; Ahn and Rosenberg, 1970). Thus the resorption and hydrolysis of thy-

roglobulin appear to be regulated by TSH. The results of this and previous studies suggest that the secretion of thyroglobulin may also be controlled by TSH. Since the effects of TSH on the resorption of thyroglobulin are mediated via the cyclic-AMP mechanism (Pastan and Wollman, 1967; Nève and Dumont, 1970; Tonoue et al., 1970; Ahn and Rosenberg, 1970), it is tempting to speculate that the same may be true of the secretion of thyroglobulin.

REFERENCES

AHN, C. S., and I. N. ROSENBERG. 1970. Proteolysis in thyroid slices: effects of TSH, dibutyryl cyclic 3',5'-AMP and prostaglandin E_1. *Endocrinology.* **86:** 870.

ALLEN, E. H., and R. S. SCHWEET. 1962. Synthesis of hemoglobin in a cell-free system. I. Properties of the complete system. *J. Biol. Chem.* **237:**760.

BACHMAN, L., and M. M. SALPETER. 1965. Autoradiography with the electron microscope. A quantitative evaluation. *Lab. Invest.* **14:**1041.

BRADLEY, A. S., and S. L. WISSIG. 1966. The anatomy of secretion in the follicular cell of the thyroid gland. III. The acute effect *in vivo* of thyrotropic hormone on amino acid uptake and incorporation into protein by the mouse thyroid gland. *J. Cell Biol.* **30:** 433.

CARO, L. G. 1962. High resolution autoradiography. II. The problem of resolution. *J. Cell Biol.* **15:**189.

CARO, L. G., and R. P. VAN TUBERGEN. 1962. High resolution autoradiography. I. Methods. *J. Cell Biol.* **15:**173.

CAVALIERI, R. R., and G. L. SEARLE. 1967. Early effects of thyrotropin on biosynthesis of thyroglobulin in the rat. *Proc. Soc. Exp. Biol. Med.* **126:**459.

DROZ, B. 1967. Synthèse et transfert des protéines cellulaires dans les neurons ganglionnaires. Etude radioautographique quantitative en microscopie électronique. *J. Microsc.* **6:**201.

FARQUHAR, M. G., and G. E. PALADE. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26:**263.

FEENEY, L., and S. L. WISSIG. 1967. Morphologic response of thyroid follicular cells stimulated in vitro with thyroid-stimulating hormone. *J. Cell Biol.* **35:**38A.

FEENEY, L., and S. WISSIG. 1971. The morphologic and physiologic behaviour of thyroid lobes from newborn rats during short periods of incubation *in vitro. Tissue and Cell.* **3:**9.

GERSH, I., and R. F. BAKER. 1943. Total protein and organic iodine in the colloid of individual follicles of the thyroid gland of the rat. *J. Cell Comp. Physiol.* **21:**213.

GERSH, I., and T. CASPERSSON. 1940. Total protein and organic iodine in the colloid and cells of single follicles of the thyroid gland. *Anat. Rec.* **78:**303.

HADDAD, A., M. D. SMITH, A. HERSCOVICS, N. J. NADLER, and C. P. LEBLOND. 1971. Radioautographic study of in vivo and in vitro incorporation of fucose-^{3}H into thyroglobulin by rat thyroid follicular cells. *J. Cell Biol.* **49:**856.

HERSCOVICS, A. 1969. Biosynthesis of thyroglobulin. Incorporation of (1-^{14}C) galactose, (1-^{14}C) mannose and (4,5-^{3}H$_2$) leucine into soluble proteins by rat thyroids *in vitro. Biochem. J.* **112:**709.

HERSCOVICS, A. 1970. Biosynthesis of thyroglobulin: incorporation of (^{3}H) fucose into proteins by rat thyroids *in vitro. Biochem. J.* **117:**411.

HODSON, S., and J. MARSHALL. 1967. Tyrosine incorporation into the rabbit retina. *J. Cell Biol.* **35:**722.

JAMIESON, J. D., and G. E. PALADE. 1967 *a.* Intracellular transport of secretory proteins in the pancreatic acinar cell. I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* **34:**577.

JAMIESON, J. D., and G. E. PALADE. 1967 *b.* Intracellular transport of secretory proteins in the pancreatic acinar cell. II. Transport to condensing vacuoles and zymogen granules. *J. Cell Biol.* **34:**597.

KALTENBACH, J. P. 1969. Methanol solubility of acid-precipitated proteins: a possible source of error in incorporation studies. *Proc. Soc. Exp. Biol. Med.* **132:**30.

KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27:**137A.

KLITGAARD, H. M., R. C. MEADE, D. K. TROCKE, H. J. PALAY, and F. L. LORSCHNEIDER. 1965. Effect of thyrotrophin on *in vivo* thyroid uptake of labelled tyrosine, arginine and iodine in the chick. *Proc. Soc. Exp. Biol. Med.* **119:**334.

LEBLOND, C. P., N. B. EVERETT, and B. SIMMONS. 1957. Sites of protein synthesis as shown by radioautography after administration of S^{35}-labelled methionine. *Amer. J. Anat.* **101:**225.

LEVINE, S. 1954. Solubilization of bovine albumin in nonaqueous media. *Arch. Biochem. Biophys.* **50:**515.

LISSITZSKY, S., M. ROQUES, J. TORRESANI, C. SIMON, and S. S. BOUCHILLOUX. 1964. Iodination and biosynthesis of rat thyroglobulin. *Biochem. Biophys. Res. Commun.* **16:**249.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265.

LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9:**409.

MACK, R. E. 1966. I^{131} metabolism in whole and sliced rat thyroid lobes. *Amer. J. Physiol.* **210:**1048.

MUNRO, H. N., and E. D. DOWNIE. 1964. Extraction of protein from tissues during treatment with organic lipid solvents. *Arch. Biochem. Biophys.* **106:**516.

NADLER, N. J., C. P. LEBLOND, and J. CARNEIRO. 1960. Site of formation of thyroglobulin in mouse thyroid as shown by radioautography with leucine-H^3. *Proc. Soc. Exp. Biol. Med.* **105:**38.

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.

NÈVE, P., and J. E. DUMONT. 1970. Effects in vitro of thyrotropin, cyclic 3',5'-AMP, dibutyryl cyclic 3',5'-AMP, and prostaglandin E$_1$ on the ultrastructure of dog thyroid slices. *Exp. Cell Res.* **63:**285.

PALADE, G. E. 1966. Structure and function at the cellular level. *J. Amer. Med. Ass.* **198:**815.

PASTAN, I., and S. H. WOLLMAN. 1967. Colloid droplet formation in dog thyroid in vitro. Induction by dibutyryl cyclic-AMP. *J. Cell Biol.* **35:**262.

PETERS, T., JR., and C. A. ASHLEY. 1967. An artefact in radioautography due to binding of free amino acids to tissues by fixatives. *J. Cell Biol.* **33:**53.

RAGHUPATHY, E., W. TONG, and I. L. CHAIKOFF. 1963. Amino acid C^{14} incorporation into protein by thyroid gland slices: effects of thyrotropic hormone injections and of hypophysectomy. *Endocrinology.* **72:**620.

SCHNEIDER, A. B., and I. H. GOLDBERG. 1965. Effect of actinomycin on TSH-induced activities of hypophysectomized rat thyroid. *Fed. Proc.* **24:**383.

SEED, R. W., and I. H. GOLDBERG. 1963. Biosynthesis of thyroglobulin: relationship to RNA-template and precursor protein. *Proc. Nat. Acad. Sci. U. S. A.* **50:**275.

SEED, R. W., and I. H. GOLDBERG. 1965. Biosynthesis of thyroglobulin. II. Role of subunits, iodination, and ribonucleic acid synthesis. *J. Biol. Chem.* **240:**764.

SELJELID, R. 1967 *a.* Endocytosis in thyroid follicle cells. II. A microinjection study of the origin of colloid droplets. *J. Ultrastruct. Res.* **17:**401.

SELJELID, R. 1967 *b.* Endocytosis in thyroid follicle cells. IV. On the acid phosphatase activity in thyroid follicle cells, with special reference to the quantitative aspects. *J. Ultrastruct. Res.* **18:**237.

SELJELID, R., A. REITH, and K. F. NAKKEN. 1970. The early phase of endocytosis in rat thyroid follicle cells. *Lab. Invest.* **23:**595.

SHIMODA, S., and M. A. GREER, 1964. Effect of temperature, pH and time of incubation on the radio-

iodine metabolism of whole rat thyroid lobes incubated *in vitro. Endocrinology.* 75:698.

SIEKEVITZ, P., and G. E. PALADE. 1960. A cytochemical study on the pancreas of the guinea pig. V. *In vivo* incorporation of leucine-1-C^{14} into the chymotrypsinogen of various cell fractions. *J. Biophys. Biochem. Cytol.* 7:619.

SMEDS, S. 1970. Protein composition of the colloid collected from single rat thyroid follicles. *Biochem. Biophys. Res. Commun.* 38:1168.

SPIRO, R. G., and M. J. SPIRO. 1965. The carbohydrate composition of the thyroglobulin from several species. *J. Biol. Chem.* 240:997.

STEIN, O., and J. GROSS. 1964. Metabolism of ^{125}I in the thyroid gland studied with electron microscope autoradiography. *Endocrinology.* 75:787.

STRUM, J. M., and M. J. KARNOVSKY. 1970. Cytochemical localization of endogenous peroxidase in thyroid follicular cells. *J. Cell Biol.* 44:655.

STRUM, J. M., and M. J. KARNOVSKY. 1971. Aminotriazole goiter. Fine structure and localization of thyroid peroxidase activity. *Lab. Invest.* 24:1.

THOMSON, J. A., and I. H. GOLDBERG. 1968. Biosynthesis of thyroglobulin and its subunits *in vivo* in the rat thyroid gland. *Endocrinology.* 82:805.

TONG, W. 1965. Thyrotropin stimulation of thyroxine synthesis in isolated thyroid cells treated with puromycin. *Endocrinology.* 76:163.

TONG, W. 1967. TSH stimulation of ^{14}C-amino acid incorporation into protein by isolated bovine thyroid cells. *Endocrinology.* 80:1101.

TONOUE, T., W. TONG, V. STOLC. 1970. TSH and dibutyryl-cyclic-AMP stimulation of hormone release from rat thyroid glands *in vitro. Endocrinology.* 86:271.

UJEJSKI, L., and R. E. GLEGG. 1955. Carbohydrates in thyroglobulin and the lens capsule. *Can. J. Biochem. Physiol.* 33:199.

VECCHIO, G., M. SALVATORE, and G. SALVATORE. 1966. Biosynthesis of thyroglobulin *in vivo*: formation and polymerization of subunits in the rat and guinea pig. *Biochem. Biophys. Res. Commun.* 25:402.

VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 25:407.

WETZEL, B. K., S. S. SPICER, and S. H. WOLLMAN. 1965. Changes in fine structure and acid phosphatase localization in rat thyroid cells following thyrotropin administration. *J. Cell Biol.* 25:593.

WHUR, P., A. HERSCOVICS, and C. P. LEBLOND. 1969. Radioautographic visualization of the incorporation of galactose-^{3}H and mannose-^{3}H by rat thyroids *in vitro* in relation to the stages of thyroglobulin synthesis. *J. Cell. Biol.* 43:289.

WILSON, B., E. RAGHUPATHY, T. TONOUE, and W. TONG. 1968. TSH-like actions of dibutyryl-cAMP on isolated bovine thyroid cells. *Endocrinology.* 83:877.

WISSIG, S. L. 1960. The anatomy of secretion in follicular cells of the thyroid gland. I. The fine structure of the gland in the normal rat. *J. Biophys. Biochem. Cytol.* 7:419.