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Modulation of the Carbohydrate Moiety of Thyroglobulin by Thyrotropin and Calcium in Fisher Rat Thyroid Line-5 Cells*

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Thyroglobulin secreted in the medium by Fisher rat thyroid line-5 (FRTL-5) cells cultured in the presence of thyroid stimulating hormone (TSH) shows a slower electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a higher density position in a CsCl gradient than thyroglobulin secreted by FRTL-5 cells cultured in the absence of TSH for 5–7 days. Such a TSH effect is much less or not evident when secreted thyroglobulin is digested with peptide *N*-glycohydrolase F or when intracellular thyroglobulin is compared. Intracellular thyroglobulin migrates faster than thyroglobulin secreted either in the presence or in the absence of TSH. Evaluation of the mannose and galactose content of thyroglobulin demonstrates that intracellular thyroglobulin has more mannose and less galactose than extracellular thyroglobulin; it also shows that TSH decreases the mannose content of thyroglobulin while increasing its galactose content. Bio-Gel P6 chromatography shows that TSH increases the complex type carbohydrate chains while decreasing the high mannose chains in the secreted thyroglobulin. High mannose type oligosaccarides were characterized by fast atom bombardment-mass spectrometry analysis.

Treatment with the calcium ionophore A23187 (5 μM) of FRTL-5 cells cultured with or without TSH causes the appearence of a "fast" migrating form of thyroglobulinin in the culture medium. Bio-Gel P6 chromatography shows that A23187 causes a dramatic decrease of the complex carbohydrate chains of the secreted thyroglobulin.

Thyroglobulin (Tg)[1] is a high molecular weight glycoprotein which is the site of synthesis of thyroid hormones. It consists of two identical subunits of 330,000 Da. During and after synthesis, the protein is modified by post-translational events such as glycosylation, phosphorylation, sulfation, and iodination. The carbohydrate moiety of Tg has been extensively studied (1–3). The great majority of the oligosaccharides of the protein are present as *N*-linked units consisting of mannose and *N*-acetylglucosamine (unit A) or mannose, *N*-acetylglucosamine, galactose, *N*-acetylneuraminic acid, and fucose (unit B). The human protein contains also *O*-linked sugars (unit C). The glycosylation of Tg involves the transfer of a $Glc_3Man_9GlcNAc_2$ oligosaccharide from a dolichol pyrophosphate carrier to the nascent protein and the subsequent removal of glucose and 1 mannose residues in the rough endoplasmic reticulum (4, 5). Additional mannose residues are removed in the Golgi complex (5) where peripheral monosaccharides of the B units are subsequently added (6).

Although the structure and the biosynthesis of the carbohydrate moiety of Tg is now well understood very little is known about their control. It has been shown that Tg secreted by primary porcine thyroid cells contains more highly branched, complex, carbohydrate chains than thyroid derived Tg. In addition, when these porcine cells were stimulated by TSH, there was an increase in the sialic acid content of Tg (7). TSH increases the incorporation of [^{3}H]mannose into thyroid glycoproteins in ovine primary thyroid cells (8), and stimulates several glycosyltransferases *in vivo* (9) and *in vitro* in porcine primary thyroid cells (10). These observations indicate a stimulatory effect of TSH on the glycosylation process *in vivo* and *in vitro*. However, it remains unclear what is the biological role of the carbohydrate moiety of Tg. We have previously reported that sequential enzymatic hydrolysis of the peripheral sugars of the complex carbohydrate chains modifies Tg structure (11) and its binding to thyroid membranes (12, 13), suggesting that the carbohydrate moiety could be implicated in thyroid hormones formation and delivery.

In recent years attention has been focused on the understanding of the intracellular transport of membrane and secretory proteins (14). The complexity of this process is suggested by genetic studies on yeast identifying at least 23 complementation groups that contribute to the transport of a membrane protein (15) and by studies that used a number of perturbants able to block the transport pathway at different stages (16–20). Information has also been accumulated on a role for calcium ions in both early and late stages of the transport pathway (21–23). Tg is the major secretory protein of the thyroid gland and follows the same general pathway described for other proteins (24).

In this report we show that, in FRTL-5 cells, TSH stimulates whereas the calcium ionophore A23187 inhibits the carbohydrate processing of Tg.

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[1] The abbreviations used are: Tg, thyroglobulin; TSH, thyroid stimulating hormone; FRTL-5, Fisher rat thyroid line; endoH, endo-β-*N*-acetyl-glucosaminidase H (EC 3.2.1.96); PNGase F, peptide *N*-glycohydrolase F (EC 3.2.2.18); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MMI, methimazole; ER, endoplasmic reticulum; FAB-MS; fast atom bombardment-mass spectrometry.

EXPERIMENTAL PROCEDURES

Materials—Coon's modified Ham's F-12 medium was from Sigma. Insulin was provided by Calbiochem (La Jolla, CA). [^{35}S]Methionine (1000 Ci/mmol), D-2-[^{3}H]mannose (10–20 Ci/mmol), and D-1-[^{3}H] galactose(5 Ci/mmol) were from Amersham Corp. Pansorbin and Pronase were from Calbiochem, Bio-Gel P6 was from Bio-Rad. EndoH and PNGase F were from Boehringer Mannheim. All other chemicals were from Sigma. BAY K 8644 was kindly supplied by Dr. Gross (Bayer).

Cell Culture—FRTL-5 cells (ATCC CRL 8305) are a continuous, cloned line of rat thyroid-differentiated cells (25). These cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and a mixture of six hormones or growth factors, *i.e.* insulin (1 μg/ml), TSH (1 mIU/ml), glycyl-histidyl-L-lysine (10 ng/ml), human transferrin (5 μg/ml), cortisone (10 nM) and somatostatin (10 ng/ml) (26). This medium is referred to as 6H medium; a similar medium lacking TSH is referred to as 5H medium.

Protein Labeling—In the experiments where TSH action was investigated, cells were labeled with [^{35}S]methionine (1000 Ci/mmol, 30 μCi/ml medium) for 3 h in 2.5 ml of methionine-free medium plus 5% calf serum and a 6H or 5H mixture. Medium was aspirated and either immunoprecipitated or ammonium sulfate precipitated (1.4–1.8 M), cells were washed three times with 3 ml of cold phosphate-buffered saline and then scraped with 2.5 ml of 0.15 M Tris, pH 7.5, plus 1% Triton X-100 and protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin, 50 μg/ml each). After 30 min on ice, cellular lysates were centrifuged at 10,000 × *g* for 30 min. Supernatants were recovered and subjected to immunoprecipitation.

In experiments where A23187 and BAY K 8644 action were investigated, FRTL-5 cells were pulse labeled with [^{35}S]methionine (80 μCi/ml) for 40 min in regular medium plus 5% calf serum and a 6H or 5H mixture. Alternatively, the cells were first incubated for 4 h in methionine-free medium plus 5% calf serum and a 6H mixture, then pulse labeled for 10 min with [^{35}S]methionine (80 μCi/ml) in the same medium and chased in regular medium with an excess of cold methionine (1000-fold) with or without A23187 or BAY K 8644. After variable chase times (from 15 min to 3 h), medium was immunoprecipitated and cells processed as above.

Carbohydrates and Protein Labeling—In experiments where TSH effect was investigated, FRTL-5 cells were double-labeled with [^{3}H] mannose (20 μCi/ml medium) and [^{35}S]methionine (10 μCi/ml medium) or [^{3}H]galactose (20 μCi/ml medium) and [^{35}S]methionine (10 μCi/ml medium) either by a 3-h incubation in medium containing one-tenth the usual concentration of glucose and methionine plus 5% calf serum and a 6H or 5H mixture, or by a 24 h incubation in regular medium. In experiments were A23187 action was investigated, FRTL-5 cells were incubated for 4 h in culture medium without glucose and methionine plus 5% calf serum and a 6H mixture, then pulse-labeled for 10 min in the same medium with [^{3}H]mannose (100 μCi/ml medium) and [^{35}S]methionine (50 μCi/ml medium) and chased in regular medium plus cold mannose and methionine (1000-fold) for 3 h. Medium was aspirated and ammonium sulfate-precipitated.

Thyroglobulin Preparations—Labeled Tg in the medium of FRTL-5 cells was purified either by simple immunoprecipitation with anti-rat Tg antibodies or by ammonium sulfate precipitation (1.4–1.8 M) followed by dialysis and immunoprecipitation. Alternatively, Tg was purified by ammonium sulfate precipitation, dialysis, and either sucrose density centrifugation on a 5–28% gradient or affinity chromatography with purified anti-rat Tg antibodies. Antisera to rat Tg were produced in rabbits as previously described (28, 29). Antibodies were purified by sodium sulfate precipitation (18–12.5%) and affinity chromatography with purified rat Tg. Immunoprecipitation of the Tg and gel electrophoresis were carried out as previously described (30, 31). Sucrose gradient centrifugation was carried out as previously described (32). Fractions comigrating with the unlabeled standard Tg were pooled, dialyzed, and lyophilized.

Isopycnic Centrifugation—10.36 g of CsCl were dissolved in 23 ml of Tris-HCl 0.01 M, pH 7.2, to give a final density of 1.30 g/ml. This solution was divided in 4 aliquots of 5.5 ml each and added to lyophilized [^{35}S]Tg (about 1 × 10^{5} cpm/sample). The isopycnic centrifugation was carried out in a Ti-50 rotor (Beckman) for 3 days at 35,000 rpm and 20 °C. At the end of the run, gradients were gently aspirated from the bottom with a peristaltic pump connected to a fraction collector. An aliquot (90 μl) of each fraction (100 μl) was counted in a Beckman liquid scintillation spectrometer.

Pronase Digestion of Tg and Chromatography of the Digest—Pronase digestion of Tg immunoprecipitated from the media of FRTL-5 cells was carried out as previously described (31). Chromatography of the Pronase digest was carried out by using a Bio-Gel P6 column (200–400 mesh) which was 150 × 0.7 cm. The column was equilibrated in 0.1 M Tris-hydrochloride, pH 8.25. Elution volumes were 0.4 ml, collected at a flow rate of 3 ml/h.

Tg Oligosaccharides Purification—3–5 mg of Tg secreted by FRTL-5 cells cultured with or without TSH was purified by ammonium sulfate precipitation and affinity chromatography and subjected to Pronase digestion (30). The Pronase digest was chromatographed on a Bio-Gel P6 column (150 × 0.7 cm). Elution volumes were 0.25 ml; the other conditions were the same as above. Fractions corresponding to the two high mannose peaks of Fig. 5, detected by the anthrone reaction (33), were separately pooled, lyophilized, and desalted by chromatography on a G-25 column (1 × 50 cm). Fractions corresponding to the single glycopeptide peak were pooled and subjected to endoH digestion. The resulting oligosaccharides were analyzed by FAB-MS.

FAB-MS Analysis of Permethylated Oligosaccharides—Oligosaccharides obtained from endoH treatment were methylated with methyliodide and methylsulfinyl-carbanion in methyl sulfoxide as previously described (34). FAB-mass spectra were recorded on a VG-analytical ZAB-2SE double focusing mass spectrometer equipped with a VG caesium gun operating at 25 KeV (2 μA). Samples were dissolved in methanol and loaded onto a glycerol-thioglycerol-coated probe tip.

Other Procedures—EndoH and neuraminidase digestion were carried out as previously described (31). PNGase F digestion (20 units/ml) was performed in 50 mM sodium phosphate, pH 7.4, 0.5% Triton X-100, 0.05% SDS, 20 mM EDTA for 24 h at 37 °C. Jack bean α-mannosidase digestion (20 units/ml) was performed in 100 mM sodium acetate, pH 5.0, for 48 h at 37 °C. The control sample always received the same treatment in the absence of the enzyme. In the double-labeling experiments, the counts of [^{35}S]methionine and [^{3}H] mannose or [^{3}H]galactose incorporated into Tg were corrected for cross-channel spillover and for decay of isotopes; disintegrations/minute calculations were made using a quenched standard curve from Beckman.

RESULTS

Thyroglobulin Secreted by FRTL-5 Cells in the Presence or in the Absence of TSH Shows a Different Electrophoretic Mobility and a Different Position in a CsCl Gradient—FRTL-5 cells synthesize and secrete Tg. Tg secreted in the culture medium by cells grown in the presence or in the absence of TSH is compared on SDS-PAGE (Fig. 1). It appears that Tg

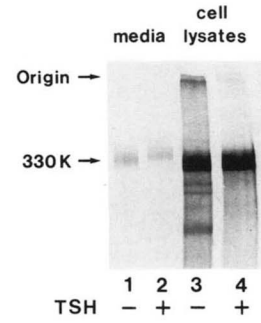

FIG. 1. **Electrophoretic mobility of Tg from FRTL-5 cells cultured with or without TSH.** FRTL-5 cells were seeded at a density of 0.5 × 10^{6} cells/60-mm tissue culture dishes. 48 h later, TSH was removed from the culture medium. After 5–7 days without TSH cells were labeled and compared with cells grown to the same density in the presence of TSH. Cells were labeled with [^{35}S]methionine for 3 h. Labeling medium (2.5 ml) was recovered, cells were rinsed, and scraped in lysis buffer (2.5 ml). Equal volumes from culture medium and cellular lysates were immunoprecipitated. A volume three times larger was immunoprecipitated from plates without TSH than from plates with TSH to correct for the effect of TSH on Tg synthesis and secretion (47). Gel electrophoresis used the Laemmli procedure (30), a 6.0% gel, and was carried out at constant current of about 20 mA for 24 h. The position of a 330-kDa molecular mass marker is noted. *K*, kDa. The additional bands faster than 330 kDa in *line 3* likely represent degradation products of Tg.

secreted by FRTL-5 cultured in the presence of TSH has a slower mobility than Tg secreted by cells cultured in the absence of TSH (compare *lane 1* with *lane 2*). This difference in electrophoretic mobility is small, and there is a certain overlap between the two bands. In contrast with secreted Tg, intracellular Tg immunoprecipitated from FRTL-5 cells cultured with or without TSH shows identical mobility (Fig. 1, compare *lane 3* with *4*) which is always higher than that of secreted Tg (Fig. 1, compare *lanes 3* and *4* with *lanes 1* and *2*). Such a TSH effect on electrophoretic mobility of Tg secreted in the medium was also evident in a primary culture from guinea pig (not shown).

In order to further investigate the molecular difference between Tg secreted in the presence or in the absence of TSH, we performed a isopycnic centrifugation in CsCl. Fig. 2 shows that the peak of Tg secreted in the presence of TSH migrates at a position of higher density compared to the peak of Tg secreted in the absence of TSH.

Thyroglobulin Secreted by FRTL-5 Cultured in the Presence or in the Absence of TSH Shows Differences in the Carbohydrate Moiety—One possibility of explaining the differences in electrophoretic mobility shown in Fig. 1 would be that they are caused by post-translational modification of Tg that is most likely iodination and/or glycosylation. A different degree of iodination or glycosylation would also explain the finding of Fig. 2 since increasing the iodine or the sialic acid content induces Tg to migrate at a position of higher density in an isopycnic centrifugation (13, 35). Following a treatment with an inhibitor of iodide organification, MMI 1 mM for 5 h, the different electrophoretic mobility between Tg secreted by FRTL-5 cultured with or without TSH and between secreted and intracellular Tg is still present (not shown). This suggests that iodination is not responsible for the changes in electrophoretic mobility that we have detected.

To test whether glycosylation plays some role in producing the different electrophoretic mobility between the "secreted" Tg in the presence or in the absence of TSH (Fig. 1, *lanes 1* and *2*), we digested Tg with PNGase F. As shown in Fig. 3 the removal of the carbohydrates from Tg increases its mobility and, more importantly, dramatically reduces or even abolishes the electrophoretic difference between the secreted Tg in the presence or in the absence of TSH.

Next we evaluated the galactose and mannose content of Tg in FRTL-5 cells cultured in a 5H mixture (time O) and at various times after addition of TSH (Fig. 4). At all times the mannose content of intracellular Tg is higher than that of secreted Tg (*panel A*), whereas the opposite is true for galactose (*panel B*). This suggests that the high mannose chains of the intracellular Tg undergo trimming, whereas galactose is added subsequently during the secretion process. TSH appears to affect both processes (mannose trimming and galactose addition).

TSH Changes the Relative Proportions of the High Mannose and Complex Carbohydrate Chains in Thyroglobulin—The data of Fig. 4 suggest that TSH increases the conversion of the high mannose chains to complex chains. To test this directly we performed an analysis of the glycopeptides of

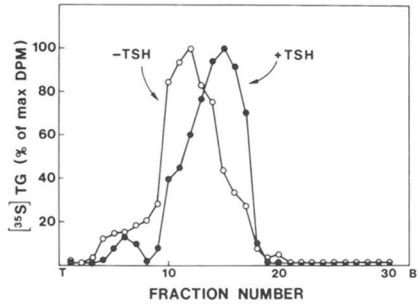

FIG. 2. **Cesium chloride centrifugation profiles of secreted Tg from FRTL-5 cultured in the presence or in the absence of TSH.** FRTL-5 cells were cultured and labeled as in Fig. 1. Culture medium (a volume three times larger from plates cultured without TSH) was ammonium sulfate precipitated (1.4–1.8 M), dialyzed, and subjected to sucrose density centrifugation on a 5–28% gradient. The pooled fractions comigrating with a 19S Tg standard were dialyzed, lyophilyzed, and subjected to a isopicnic centrifugation in a CsCl solution 2.68 molal with a starting density of 1.30 g/cm^3. Density increases from left (*top*) to right (*bottom*). Shown is a representative of three different experiments each with duplicate tubes.

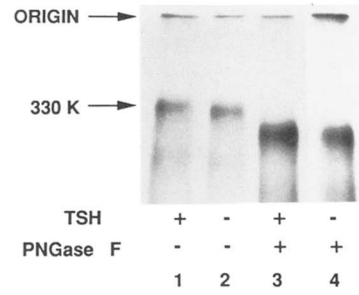

FIG. 3. **Digestion with PNGase F greatly reduces the difference in electrophoretic mobility between Tg secreted in the presence and in absence of TSH.** FRTL-5 cells were plated, cultured, and labeled as in Fig. 1. Secreted Tg was immunoprecipitated and subjected to PNGase F digestion as described under "Experimental Procedures." The control samples received the same incubation without the enzyme. Gel electrophoresis was carried out as in Fig. 1. *Lanes 1–3* were exposed 24 h; *lane 4* was exposed 48 h.

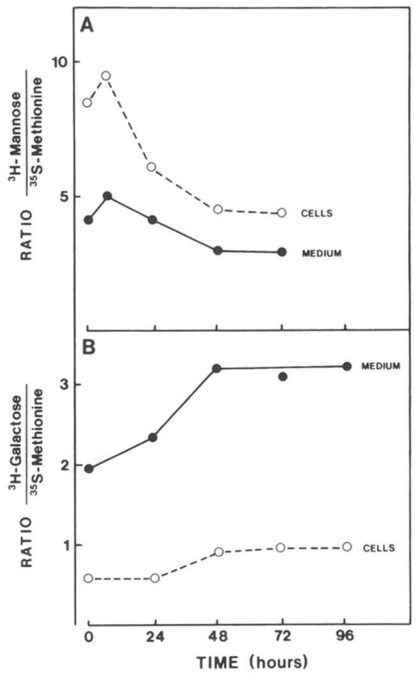

FIG. 4. **Quantitation of the [^{3}H]mannose and [^{3}H]galactose in intracellular and secreted Tg during a TSH stimulation of FRTL-5 cells.** FRTL-5 cells were plated and "starved" from TSH as in Fig. 1. Then TSH (1 mIU/ml) was added (time 0). At various times after TSH addition cells were double labeled with [^{3}H]mannose and [^{35}S]methionine or [^{3}H]galactose and [^{35}S]methionine for 3 h (see "Experimental Procedures"). The times indicated in the figure include the labeling times. The culture medium and cell lysates were immunoprecipitated and loaded on a 7.5% Laemmli gel (30). Areas of the gel comigrating with the unlabeled 330-kDa Tg marker were excised, digested for 48 h at 50 °C in 30% (w/v) H_2O_2, and counted in a Bekman scintillation spectrometer.

secreted Tg by gel filtration (Fig. 5). FRTL-5 cells were double-labeled with [^{3}H]galactose and [^{35}S]methionine or [^{3}H] mannose and [^{35}S]methionine, and secreted Tg was purified, digested with Pronase, and chromatographed.

In the absence of TSH (Fig. 5, *panel A*), the glycopeptides separate into three major peaks; the first one (fractions 38–50, correspondig to about 30% of the total dpm) is eluted just after the void volume: most likely it represents glycopeptides that bear complex type carbohydrate chains since the peak is endoH resistant (not shown) and is selectively labeled with [^{3}H]galactose. The second and third peaks (fractions 51–70, corresponding to about 70% of the total dpm) represent glycopeptides with high mannose chains because they are endoH sensitive (not shown), and they are not labeled with [^{3}H]galactose. The presence of TSH in the culture medium (*panel B*) increases the first peak to about 50% of the total dpm and decreases the second and third peaks to about 50% of the total dpm. In these experiments we used a double-labeled Tg since we could use the [^{35}S]methionine label to check the reproducibility of the Pronase hydrolysis that indeed seems reproducible (Fig. 5, compare [^{35}S]methionine elution profiles in *panels A* and *B*).

The glycopeptides corresponding to each of the two high mannose peaks, purified by chromatography on a Bio-Gel P6 column (see "Experimental Procedures"), were digested with endoH and the released oligosaccharides were characterized by FAB-MS. In the absence of TSH, the mass spectrum of the first high mannose peak (peak around fraction 51, Fig. 5, *panel A*), after endoH and methylation, showed signals exclusively corresponding to $(MH)^+$ and $(MNa)^+$ for permethylated $Man_9GlcNAc$ (*m/z* 2128 and 2150, not shown). The mass spectrum of the second high mannose peak (peak around fraction 58, Fig. 5, *panel A*) showed signals corresponding to the structure $Man_9GlcNAc$ (*m/z* 2128 and 2150) and $Man_8GlcNAc$ (*m/z* 1924 and 1946); there were also weak signals due to traces of $Man_7GlcNAc$ (*m/z* 1720 and 1742) and $Man_6GlcNAc$ (*m/z* 1516 and 1538) (Fig. 6, *panel A*).

In the presence of TSH, FAB-MS analysis of the permethylated first high mannose peak (peak around fraction 51, Fig. 5, *panel B*) exhibited ions corresponding to the structure $Man_9GlcNAc$ (*m/z* 2128 and 2150, not shwon). The spectrum of the second high mannose peak (peak around fraction 57, Fig. 5, *panel B*) contained signals corresponding to structures $Man_9GlcNAc$, $Man_8GlcNAC$, $Man_7GlcNAC$, $Man_6GlcNAc$, $Man_5GlcNAc$ (Fig. 6, *panel B*).

The TSH Effect on Carbohydrate Chains of Tg Is Also Present in Swansonine-treated Cells—The finding of an increased capability to process the carbohydrate chains in the presence of TSH is strengthened by the experiment shown in Fig. 7. In this experiment FRTL-5 cells cultured for 7 days with or without TSH were treated with swainsonine, an inhibitor of mannosidase II, and labeled with [^{3}H]mannose. Secreted Tg was purified, digested with Pronase, and chromatographed. In the presence of swainsonine the elution profile of Tg glycopeptides shows two major peaks: the first one elutes around fraction 47, and the second one, very broad, has a maximum at fraction 51 (*panel A*). Furthermore, Tg secreted in the presence of swainsonine migrates faster on SDS-PAGE than Tg secreted in the absence of the drug. This may very likely be due to the change in its glycosylation (compare the elution profiles of Fig. 5 and of Fig. 7, *panel A*). The presence of TSH in the culture medium causes an increase of the first peak and a decrease of the second one. When the endoH-released oligosaccharides were studied (Fig. 7, *panel B*) the TSH effect is more evident probably because there is some heterogeneity in the peptide moiety of the glycopeptides of *panel A*. In order to elucidate the structure (hybrid type or high mannose) of the two major peaks released

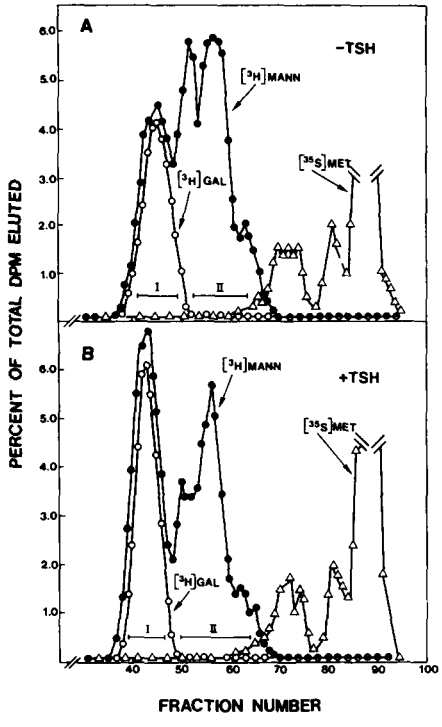

FIG. 5. **Effect of TSH on the elution profiles of Pronase-digested Tg secreted in the medium by FRTL-5 cells.** FRTL-5 cells were seeded at a density of 1.5×10^6 cells/100-mm tissue culture dishes. 48 h later, TSH was removed from the medium. After 5–7 days without TSH, cells were labeled and compared with cells grown to the same density in the presence of TSH. The cells were dually labeled with [^{3}H]mannose and [^{35}S]methionine or [^{3}H]galactose and [^{35}S]methionine for 24 h as described under "Experimental Procedures." The medium was ammonium sulfate precipitated, dialyzed, immunoprecipitated, and subjected to Pronase digestion. After desalting, the material (about 60,000 dpm for [^{3}H]mannose and [^{3}H] galactose, about 40,000 for [^{35}S]methionine) was divided in 2 aliquots, mock digested (*panels A* and *B*) or digested (not shown) with endoH and chromatographed on a Bio-Gel P6 column (200–400 mesh). The column was 150×0.7 cm.; the fractions were 0.4 ml and the flow rate was 3 ml/h. The recoveries were in all cases graeter than 95%.

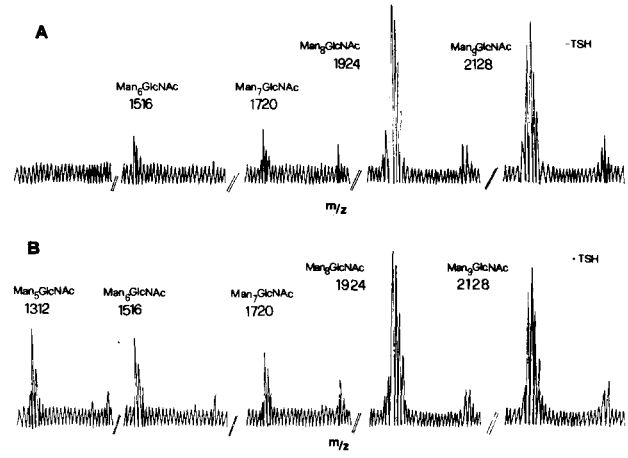

FIG. 6. **Partial positive-ion FAB-mass spectra of permethylated high mannose oligosaccharides of Tg in the absence (*A*) and in the presence (*B*) of TSH.** The oligosaccharides were obtained by endoH digestion of the two high mannose peaks of Fig. 5 as described under "Experimental Procedures." The ions corresponding to $(MH)^+$ for Man_9, Man_8, Man_7, Man_6,and Man_5 are labeled.

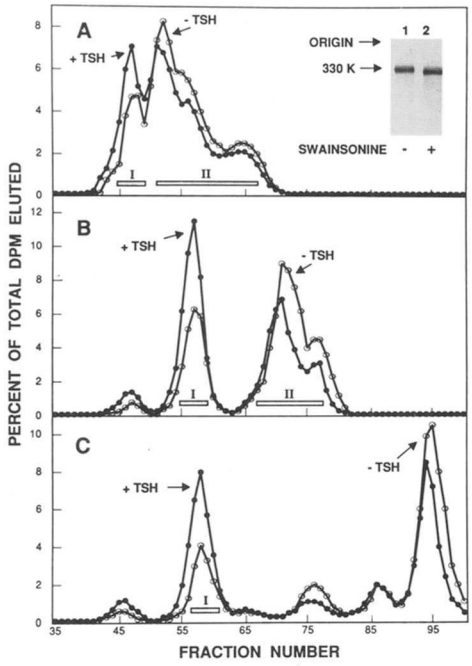

FIG. 7. **Effect of swansonine on the elution profiles of Pronase-digested Tg secreted in the medium by FRTL-5 cells.** FRTL-5 were cultured as in Fig. 5. Swainsonine (8 μg/ml) was added 2 h before labeling. Cells were dually labeled with [^{3}H]mannose and [^{35}S]methionine for 24 h as described under "Experimental Procedures." The culture medium was ammonium sulfate precipitated, dialyzed, immunoprecipitated, and subjected to Pronase digestion. After desalting, the material (about 60,000 dpm for [^{3}H]mannose and 40,000 for [^{35}S]methionine) was divided in 3 aliquots, mock digested (*panel A*), or digested with endoH (*panel B*) or with endoH first and then with α-mannosidase (*panel C*), and chromatographed on a Bio-Gel P6 column (200–400 mesh). The column size, elution buffer, and elution rate were the same as in Fig. 5. In all cases recoveries were greater than 95%. The *inset* of *panel A* shows the electrophoretic mobility of Tg secreted by FTRL-5 cells cultured in a 6H mixture treated or not treated with swainsonine (8 μg/ml). The samples were immunoprecipitated and subjected to a 7.5% Laemmli gel.

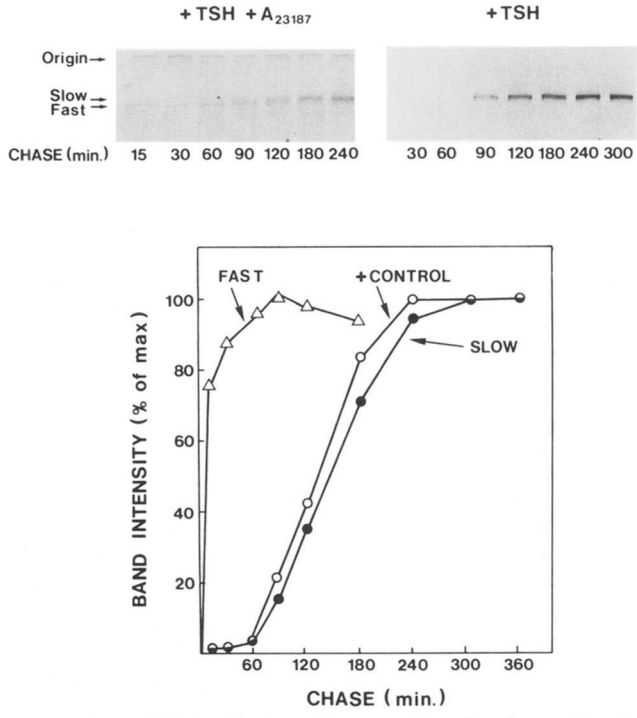

FIG. 8. **A23187 effect on Tg secretion in the presence of TSH.** FRTL-5 cells routinely grown in 5% calf serum and a 6H mixture were seeded at a density of 1.5×10^6 cells/60-mm tissue culture dishes. After 3 days cells were pulse labeled for 40 min with [^{35}S]methionine and chased either in the presence or in the absence of A23187 (5 μM). At various chase times secreted Tg was immunoprecipitated and subjected to a 7.5% Laemmli gel. The gel of the controls (without A23187) was exposed 1 week, compared with 2 days for the gel of A23187-treated semples, to show the absence of Tg at early chase times.

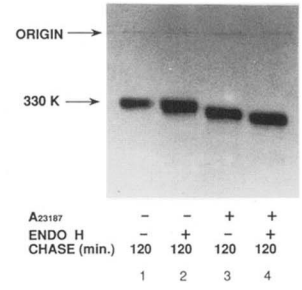

FIG. 9. **Tg secreted in the presence of A23187 is more sensitive to endoH digestion than Tg secreted in the absence of the ionophore.** FRTL-5 cells were plated and cultured as in Fig. 8. They were pulse labeled for 10 min with [^{35}S]methionine as described under "Experimental Procedures" and chased for 120 min with or without A23187. Secreted Tg was immunoprecipitated and subjected to endoH digestion; the control samples received the same incubation without the enzyme. Gel electrophoresis used a 7.5% Laemmli gel.

by endoH digestion (*peaks I* and *II*, Fig. 7, *panel B*), the resultant material was subjected to an α-mannosidase digestion and subsequent chromatography. Whereas peak II disappears under α-mannosidase digestion with almost all of the label recovered as free mannose (Fig. 7, *panel C*), peak I was only slightly shifted and reduced. These results suggest that peak I represents hybrid type oligosaccarides whereas peak II represents high mannose oligosaccarides.

A23187 Affects Both Thyroglobulin Secretion and Carbohydrate Processing—The role of calcium in both early and late stages of the transport pathway is well known (21–23). To investigate whether calcium plays a role in Tg secretion and processing in FRTL-5 cells, we performed experiments with the calcium ionophore A23187.

When FRTL-5 cells, grown in 5% calf serum and a 6H mixture, were pulse labeled for 40 min with [^{35}S]methionine and chased in the presence of A23187 (5 μM) a band migrating faster than Tg appears in the media at early chase times. This band represents a fast migrating form of Tg since it is immunoprecipitated with anti-Tg antibodies. At later chase times (90 min), coincident with the appearence in the media of Tg in the controls, the "normal" migrating form of Tg appears (Fig. 8). This effect was also present in FRTL-5 cells cultured in the absence of TSH for 5–7 days (not shown).

With a short [^{35}S]methionine pulse (10 min), the Tg secreted in the medium is a quite homogeneous population of the "fast" band secreted when a 40-min pulse was performed (Fig. 9, *lane 3*). Tg secreted in the presence of A23187 is perhaps more sensitive to endoH digestion than Tg secreted in the absence of the ionophore (Fig. 9, compare the difference in mobility between *lanes 1* and *2* with the difference between *lanes 3* and *4*). This may suggest that A23187 inhibits the processing of the carbohydrate chains of Tg.

Therefore, an analysis of the carbohydrate chains of Tg were performed. FRTL-5 cells were pulse labeled for 10 min with [^{35}S]methionine and [^{3}H]mannose and chased for 180 min either in the presence or in the absence of A23187. Tg secreted in the medium was purified, digested with Pronase, and chromatographed. A23187 causes (Fig. 10) a dramatic decrease of the first peak containing complex type carbohy-

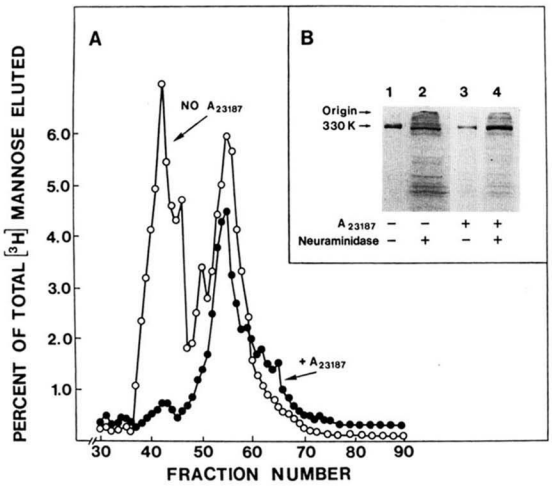

FIG. 10. **Effect of A23187 on the elution profiles of Pronase-digested Tg secreted in the medium by FRTL-5 cells.** *Panel A*, FRTL-5 cells routinely cultured in 5% calf serum and a 6H mixture were plated at a density of 4×10^6 cells/100-mm tissue culture dishes. After 3 days cells were pulse labeled for 10 min with [^{3}H]mannose and [^{35}S]methionine as described under "Experimental Procedures" and chased for 3 h with or without A23187 (5 μM). Culture medium was ammonium sulfate precipitated, dialyzed, immumoprecipitated, and subjected to Pronase digestion. After desalting, the material (about 100,000 dpm for [^{3}H]mannose and 40,000 for [^{35}S]methionine) was divided in 2 aliquots, mock digested (*panel A*) or digested with endoH (not shown), and chromatographed on a Bio-Gel P6 column (as in Fig. 5). In all cases the recoveries were greater than 95%. *Inset B* shows the electrophoretic mobility of a small aliquot of the samples from *panel A* (one-tenth) which after being ammonium sulfate precipitated and dialyzed were digested with neuraminidase and subjected to a 7.5% Laemmli gel (30).

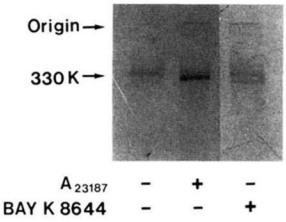

FIG. 11. **BAY K 8644, a specific calcium channel activator, reproduces in part the A23187 effect on electrophoretic mobility of Tg.** FRTL-5 cells were plated and cultured as in Fig. 8. They were pulse labeled for 10 min with [^{35}S]methionine as described under "Experimental Procedures" and chased for 40 min with A23187 (5 μM) or BAY K 8644 (1 μM). Secreted Tg was immunoprecipitated and subjected to a 7.5% Laemmli gel. The greater amount of Tg evident in the *A23187 lane* is probably due to the minor wideness of the lane and to the relatively short chase time. All lanes were from the same gel.

drate chains (the first peak was endoH resistant and the second was endoH sensitive as in Fig. 5, not shown). *Inset B* of Fig. 10 shows that Tg from cells treated with A23187 lacks neuraminidase sensitivity, suggesting that in this condition Tg has very few if any sialic acid residues in its carbohydrate moiety. In the Tg secreted in the presence of A23187 we found an increased ratio [^{3}H]mannose/[^{35}S]methionine (about 30%). This result, taken togheter with those of Fig. 10, strongly suggests that the high mannose units of Tg undergo a less extensive processing in the presence of the ionophore. These findings raise the question of whether Tg secretion under the effect of A23187 takes place along the normal or a "novel" secretory pathway.

In order to examine this question, Tg secretion was blocked by monensin which causes the Golgi apparatus to become destabilized in many cells. In open follicles from pig thyroid it has been shown that monensin does not inhibit either the transport of Tg from the site of monensin-induced arrest in the Golgi complex to the apical surface or the exocitosis (36). In FRTL-5 cells the addition of monensin at the beginning of the chase, after a 10-min pulse, inhibits Tg secretion by 90%, and A23187 is completely unable to overcome this block (not shown). The same results were obtained by chasing FRTL-5 cells at 20 °C (not shown). This suggests that there are no dramatic changes in the secretion pathway followed by Tg in the presence of A23187. The specific calcium channel activator BAY K 8644 (37) partially reproduces the effect of A23187 (Fig. 11). In its presence, Tg secreted in the medium migrates as a doublet on SDS-PAGE. The lower band migrates as the Tg secreted in the presence of A23187, while the upper band migrates as the Tg of the control.

DISCUSSION

In this study we show that, in FRTL-5 cells, secreted Tg has a slower electrophoretic mobility in SDS-PAGE than intracellular Tg. The presence of TSH in the culture medium further decreases the mobility of secreted Tg. A different glycosylation could be responsible for these changes in mobility (20), and indeed we show that treatment with PNGase F dramatically reduces or even abolishes the different mobility between Tg secreted with or without TSH. The effect of TSH is directed toward stimulation of the processing of the high mannose chains to complex chains. However, this maturation is taking place also in its absence, although to a lower extent.

Recently, it has been reported (38), using prymary porcine thyroid cells, that extracellular Tg is slower on SDS-PAGE and is less sensitive to endoH digestion than intracellular Tg. We noticed the same differential sensitivity to endoH for intracellular and secreted Tg. These results confirm, using another system, what we have found in FRTL-5 cells.

Ronin *et al.* (7) did not find any variation in the relative proportion of complex and high mannose chains in porcine thyroid cells under TSH stimulation. We believe that this difference could depend on the different conditions of culture or the different system used.

Dunn and Ray (39), following an *in vivo* TSH administration in the guinea pig, found a change in the electrophoretic pattern of Tg reminiscent of what we have found in FRTL-5 cells. In response to TSH, there was an increase of the slowest bands and a concomitant decrease of the fastest bands of the pattern. It may be that some change in the carbohydrate chains was present, although undetected perhaps because the large Tg pool present in animals.

It has been reported that TSH, both *in vivo* (9) and *in vitro* in porcine primary thyroid cells (10), increases the activity of several glycosyltransferases. Our results could suggest that TSH stimulates, in FRTL-5 cells, the mannosidases. Thus the FAB-MS spectrum of the high mannose glycopeptides peaks obtained in the presence of TSH compared with that obtained in the absence of TSH reveals a decisive shift to the lightest structures. In particular the structure $Man_5GlcNAc$, completely absent without TSH, is clearly identified in the presence of TSH by the signals at *m/z* 1312 and 1334. In addition, even when the mannosidase II is inhibited by the addition of swainsonine, there is an enhanced capability to process high mannose chains in the presence of TSH. This would indicate that the enzymes acting before mannosidase II are TSH-responsive.

In this study we demonstrate that a brief treatment of FRTL-5 cells with A23187 affects the glycosylation of Tg secreted in the medium. Several other agents have been reported to affect the glycosylation of secretory and membrane proteins. These agents include weak bases (40), ionophores such as monensin (18), reduced extracellular pH (19),

and Tris (20). All these agents also dramatically inhibit the transport of the secretory and membrane proteins to the extracellular medium and to the cell surface. On the other hand, A23187 does not have any significant effect on the amount of Tg secreted suggesting that its mechanism of action is different from that of the previously used agents. In addition, a specific calcium channel activator, BAY K 8644 (37), reproduces, at least partially, the effect of A23187 on the electrophoretic mobility of Tg. Therefore calcium, and not other ions, seems to be responsible for the effects we have observed.

Recently, evidences have been accumulated on the role of calcium ions in transport of proteins from the ER. Reticuloplasmins are secreted in the medium when murine fibroblasts are treated with A23187 (41). Yeast strains carrying a mutation in a gene encoding a calcium ATPase (pmr 1 mutant) that is believed to pump calcium into an intracellular storage compartment secrete foreign proteins such as urokinase that in the wild type are trapped in the ER. In addition, the yeast secretory protein invertase secreted from the mutant is incompletely glycosilated lacking the mannose residues normally added in the yeast Golgi apparatus (42, 43). Therefore, A23187 seems to cause in mammalian cells the same effects that follow an impairing of the function of a yeast calcium ATPase. This suggests that the loss of calcium from the ER is responsible both for the secretion of resident ER proteins and for the abnormal glycosylation of secretory proteins.

Our data argue against dramatic modifications of the normal secretory route followed by Tg in the presence of A23187, since the ionophore does not cause Tg to overcome the monensin and 20 °C blocks of secretion. Therefore, in the presence of A23187, there could be some subtle change in the secretory pathway, perhaps in the ER to Golgi transport for which calcium is essential (21). It is unlikely that A23187 directly inhibits the activity of the enzymes involved in carbohydrate processing since reticuloplasmins secreted under the action of A23187 bear complex carbohydrate chains (41).

It is possible that the effects of TSH and A23187 on the carbohydrate moiety of Tg are the result of an opposite action of these agents on intracellular calcium. This seems unlikely given the very different time course of the actions of the two agents. Thus while TSH requires several hours to stimulate carbohydrate processing, A23187 inhibits it in few minutes. In addition, it has been reported that TSH and cAMP do not elicit any change in the cytosolic calcium concentration in dog primary cells (45) and in FRTL-5 cells (46).

Our results demonstate that, in FRTL-5 cells, TSH and calcium exert opposite actions on the maturation of the high mannose chains to complex chains of Tg. Previous studies that used the sequential enzymatic hydrolysis of the peripheral sugars of the complex carbohydrate chains have shown that these sugars play a role in Tg structure (11) and thyroid membrane binding (12, 13). Thus it would be of interest to investigate if this modulation of the processing of the carbohydrate moiety of Tg has some consequence on the process of thyroid hormone formation and delivery.

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REFERENCES

1. Spiro, R. G. (1965) *J. Biol. Chem.* **240,** 1603–1610
2. Fukuda, M., and Egomi, F. (1971) *Biochem. J.* **123,** 407–415
3. Arima, T., Spiro, M. J., and Spiro, R. G. (1972) *J. Biol. Chem.* **247,** 1825–1835
4. Ronin, C., and Bouchilloux, S. (1976) *Biochim. Biophys. Acta* **428,** 445–455
5. Godelaine, D., Spiro, M. J., and Spiro, R. G. (1981) *J. Biol. Chem.* **256,** 10161–10168
6. Ring, P., Bjorkman, U., and Ekholm, R. (1987) *Cell Tissue Res.* **250,** 149–156
7. Ronin, C., Fenouillet, E., Hovsepian, S., Fayet, G., and Fournet, B. (1986) *J. Biol. Chem.* **261,** 7287–7293
8. Eggo, M. C., and Burrow, G. N. (1982) *Endocrinology* **111,** 1663–1665
9. Spiro, M. J. (1980) *Arch. Biochem. Biophys.* **202,** 35–42
10. Franc, J. L., Hovsepian, S., Fayet, G., and Bouchilloux, S. (1984) *Biochem. Biophys. Res. Commun.* **118,** 910–915
11. Shifrin, S., Consiglio, E., and Kohn, L. D. (1983) *J. Biol. Chem.* **258,** 3780–3786
12. Consiglio, E., Salvatore, G., Rall, J. E., and Kohn, L. D. (1979) *J. Biol. Chem.* **254,** 5065–5076
13. Consiglio, E., Shifrin, S., Yavin, Z., Ambesi-Impiombato, F. S., Rall, J. E., Salvatore, G., and Kohn, L. D. (1981) *J. Biol. Chem.* **256,** 10592–10599
14. Burgess, T. L., and Kelly, R. B. (1987) *Annu. Rev. Cell. Biol.* **3,** 243–294
15. Novick, P., Field, C., and Schekman, R., (1980) *Cell* **21,** 205–215
16. Saraste, J., Palade, G. E., and Farquhar, M. G. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6425–6429
17. Matlin, K. S. (1986) *J. Biol. Chem.* **261,** 15172–15178
18. Griffiths, G., Quinn, P., and Warren, G. (1983) *J. Cell. Biol.* **96,** 835–850
19. Matlin, K. S., Skibbens, J., and McNeil, P. L. (1988) *J. Biol. Chem.* **263,** 11478–11485
20. Oda, K., Ogata, S., Koriyama, Y., Yamada, E., Mifune, K., and Ikehara, Y. (1988) *J. Biol. Chem.* **263,** 12576–12583
21. Beckers, C. J. M., and Balch, W. E. (1989) *J. Cell. Biol.* **108,** 1245–1256
22. Burgoyne, R. D. (1987) *Nature* **328,** 112–113
23. De Curtis, I., and Simons, K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8052–8056
24. Ekhlom, R. (1981) *Mol. Cell. Endocrinol.* **24,** 141–163
25. Ambesi-Impiombato, F. S. (Aug. 26, 1986) U. S. Patent 4608341
26. Ambesi-Impiombato, F. S., Parks, L. A. M., and Coon, H. G. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 4355–4359
27. Nitsch, L., and Wollman, S. H. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 472–476
28. Mullin, B. R., Levinson, R. E., Friedman, A., Henson, D. E., Winand, R. J., and Kohn, L. D. (1977) *Endocrinology* **100,** 351–366
29. Fontana, S., and Rossi, G. (1980) in *Autoimmune Aspects of Endocrine Disorders* (Pinchera, A., Doniach, D., Fenzi, G. F., and Baschieri, L, eds) pp. 147–152, Academic Press, New York
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685
31. Consiglio, E., Acquaviva, A. M., Formisano, S., Liguoro, D., Gallo, A., Vittorio, T., Santisteban, P., De Luca, M., Shifrin, S., Yeh, H. J. C., and Kohn, L. D. (1987) *J. Biol. Chem.* **262,** 10304–10314
32. Cortese, F., Schneider, A. B., and Salvatore, G. (1976) *Eur. J. Biochem.* **68,** 121–129
33. Spiro, R. G. (1966) *Methods Enzymol.* **8,** 3–26
34. Hakomori, S. (1964) *J. Biochem. (Tokyo)* **55,** 205–208
35. Schneider, A. B., and Edelhoch, H. (1971) *J. Biol. Chem.* **246,** 6592–6598
36. Ring, P., Bjorkman, U., Johanson, V., and Ekholm, R. (1987) *Cell Tissue Res.* **248,** 153–160
37. Schramm, M., Thomas, G., Towart, R., and Franckowiak, G. (1983) *Nature* **303,** 535–537
38. Arvan, P., and Lee, J. (1991) *J. Cell Biol.* **112,** 365–376
39. Dunn, J. T., and Ray, S. C. (1975) *J. Biol. Chem.* **250,** 5801–5807
40. Thorens, B., and Vassalli, P. (1986) *Nature* **321,** 618–620
41. Booth, C., and Koch, G. L. E. (1989) *Cell* **59,** 729–737
42. Sambrook, J. F. (1990) *Cell* **61,** 197–199
43. Rudolph, H. K., Antebi, A., Fink, G. R., Buckley, C. M., Dorman, T. E., LeVitre, J., Davidow, L. S., Mao, J., and Moir, D. T. (1989) *Cell* **58,** 133–145
44. Sheela Rani, C. S. S., Boyd, A. E., III, and Field, J. B. (1985) *Biochem. Biophys. Res. Commun.* **131,** 1041–1047
45. Corda, D., Marcocci, C., Kohn, L. D., Axelrod, J., and Luini, A. (1985) *J. Biol. Chem.* **260,** 9230–9236
46. Santisteban, P., Kohn, L. D., and Di Lauro, R. (1987) *J. Biol. Chem.* **262,** 4048–4052