Relationship of Glycosylation to de Novo Thyroid-stimulating Hormone Biosynthesis and Secretion by Mouse Pituitary Tumor Cells*

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We have studied the de novo biosynthesis and secretion of thyroid-stimulating hormone (TSH) in dispersed mouse pituitary tumor cells. After labeling with [35S]methionine or various 3H-carbohydrates, cell lysates and media were immunoprecipitated with antisera to either TSH-a or TSH-b, and the products analyzed by sodium dodecyl sulfate gradient gel electrophoresis. During a 10-min pulse, the predominant [35S]-labeled a form was of apparent Mr = 18,000 with a second component of Mr = 21,000, the latter corresponding to standard pituitary a. When the labeled pulse was followed by variable chase periods with excess unlabeled methionine, the 18,000 a form was converted progressively to the 21,000 mature form, implying a precursor-product relationship between the two. The initial [35S]-labeled b form of Mr = 18,000 was not processed further, but combined selectively with the excess mature a form (Mr = 21,000) within 10 to 30 min of the chase period. TSH and excess free a (Mr = 23,000), but not free b, were secreted between 60 to 240 min of the chase. During continuous labeling, the ratio of the two intracellular a forms remained relatively constant. The [35S]methionine a/b ratio was 4.0 and the molar a/b ratio estimated to be 2.7; there was no significant degradation of either labeled subunit for up to 240 min. After 120 min of continuous labeling, the intracellular a form of Mr = 21,000 incorporated more [3H]glucosamine and galactose, similar amounts of mannose, and less fucose, compared to the a form of Mr = 18,000. Inhibition of subunit glycosylation by tunicamycin resulted in the secretion of a new form of Mr = 11,000, whose combination with b subunit was inhibited. These data suggest that the b subunit is limiting in TSH biosynthesis and that specific glycosylation may be required for a/b subunit combination but not secretion.

Thyroid-stimulating hormone (TSH) is composed of two glycosylated, noncovalently linked subunits, a and b. Within a species, the TSH-a subunit is virtually identical with that of pituitary and placental gonadotropins, while the TSH-b subunit is unique and confers hormonal specificity (1). Recent studies of TSH biosynthesis in cell-free systems (2-6) have indicated that the a and b subunits are synthesized from separate messenger RNA. The initial synthesized forms appear to be nonglycosylated "presubunits." However, only pre-a has been characterized chemically and shown to contain an NH2-terminal hydrophobic extension peptide (5).

In contrast to these detailed studies of TSH biosynthesis in cell-free systems, there has been little attention to de novo TSH biosynthesis in intact cell systems. Initial biosynthetic studies from our laboratory demonstrated a precursor b form of apparently small size by gel chromatography, but no precursor TSH-b form was identified (7).

In the present study, we examine the de novo biosynthesis of TSH in dispersed mouse pituitary tumor cells, using both pulse-chase and continuous labeling techniques combined with improved analytic methods that permit identification of both free and combined subunits. The relationship of glycosylation to TSH biosynthesis and secretion has been studied by comparing the incorporation of [35S]methionine and various 3H-carbohydrates before and after the addition of tunicamycin, an inhibitor of protein glycosylation (8, 9).

EXPERIMENTAL PROCEDURES

Thyrotropic Tumor Cell Cultures—Pituitary thyrotropic tumors (NIH 102 and 103) were induced and transplanted in hypothyroid female LAF, mice as described previously (10). Cells from one to three tumors were dispersed enzymatically by the method of Vale et al. (11) and preincubated overnight at 37°C in moist 5% CO2, 95% air, in sterile flasks containing 200 ml of Dulbecco's Modified Eagle's Medium (DMEM medium) supplemented with 10% (v/v) hypothyroid calf serum (Rockland Farms), 10 mm Hepes, 2 mm glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. After preincubation, cells (2 to 5 x 109) were washed two times in the appropriate experimental medium, and identical aliquots added to either 25- or 75-cm2 culture dishes (Falcon Plastics) containing a final volume of either 2.5 or 10 ml and 10 to 109 cells in suspension culture.

Tunicamycin—Tunicamycin prepared by Dr. John Douros, Natural Products Branch, National Cancer Institute, was generously supplied by Dr. Ray Ruddon, NCI Frederick Cancer Research Center. Various concentrations of this drug up to 5 μg/ml were added to certain culture flasks at the beginning of the incubation period immediately prior to the addition of labeled precursors.

Incorporation of Labeled Precursors—Cells were incubated at 37°C in moist 5% CO2, 95% air for various periods in either serum-free, methionine-free medium containing 25 μCi/ml (approximately 2 x 1010) of L-[35S]methionine (Amersham/Searle, 800 to 1200 Ci/mmol), or serum-free medium containing 20 μCi/ml of either D-[6-3H]glucosamine, D-[2-3H]mannose, or L-[6-3H]glucose (Amersham/Searle, 0.5 to 20 Ci/mmol). In certain cases 10% hypothyroid serum was also added to the medium and this did not change appreciably the incorporation of radioactivity into TSH, which was linear up to 24 h.

In continuous labeling experiments, cultures were terminated by the addition of excess unlabeled methionine or carbohydrate to a final concentration of 0.01% (w/v) and chilling of the dishes on ice. Medium and suspended cells were removed immediately, plates were rinsed with additional 2 ml of cold media and gently scraped with a rubber policeman to remove attached or adsorbed cells. This rinse was combined with the removed media and cells, and centrifuged at 1,200 x g for 5 min at 4°C. Medium was removed and the cell pellet rinsed.

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** The abbreviations used are: TSH, thyroid-stimulating hormone; CG, choriogonadotropin; LH, luteinizing hormone; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(Received for publication, November 26, 1979)
Fig. 1. Immunoprecipitation of [35S]methionine-labeled TSH-α and TSH-β synthesized by mouse thyrotropic tumor cells during pulse-chase study. A, B, cell lysate (intracellular) radioactivity; C, medium (extracellular) radioactivity. Analysis was by SDS-gradient polyacrylamide slab gels having a 1-cm stacking and 9-cm resolving gel. Details of immunoprecipitation and gel analysis are described under ‘‘Experimental Procedures’’ and specificity of antisera in Table I. At each time, cells had been exposed to a constant 10-min pulse of [35S]methionine followed by a chase of 30,000-fold excess unlabeled methionine for the various times indicated (0 to 240 min). Solid lines, unshaded bars (anti-bLH-α); dashed lines, shaded bars (anti-bTSH-β); crossed lines (nonimmune serum). Internal molecular weight markers (M = 12,000 to 66,000, see ‘‘Experimental Procedures’’) identified by Coomassie brilliant blue stain were included in each lane of slab gels and used to align figures. Also indicated are the apparent M, of standard rat LH-α and TSH-β as well as that of mouse tumor pre-α (3, 5) which were determined in separate experiments. The total volume of cell lysate (A and B) was 2 ml and the volume immunoprecipitated was 0.24 ml; total volume of medium (C) was 12 ml and the volume precipitated was 0.64 ml. To normalize with respect to the total radioactivity in A and B, counts per min in C should be multiplied by 2.

Once with 10 ml of additional media; the cell pellet was centrifuged and the rinse discarded. The cell pellet was then lysed by the addition of 1 ml of a solution containing 200 units of aprotinin (Trasylol, Calbiochem) and 2% (v/v) Triton X-100 (Research Products International) followed by vigorous blending on a Vortex mixer for 15 s. Media and cell lysates were frozen and stored at −20°C.

In “pulse-chase” experiments, cultures were incubated with [35S]methionine for 10 min (“pulse”) after which further de novo incorporation was inhibited by the addition of 30,000-fold excess of unlabeled methionine at a final concentration of 6.7 × 10−4 M. Cultures were then reincubated at 37°C for variable periods (“chase”) and processed as described above.

Immunoprecipitation—Media and cell lysates were thawed and centrifuged at 1,450 × g for 15 min at 4°C. Lysates were further centrifuged at 100,000 × g for 2 h. Duplicate aliquots of media or cell lysates (50 to 800 μl) were incubated in a total volume of 1 ml containing 0.15 M sodium chloride, 0.02 M Tris-HCl, 0.01 M EDTA, 1% (w/v) Triton X-100, and 100 units/ml of aprotinin, pH 7.6. To each of the replicates was added 10 μl of either rabbit anti-bovine LH-α, anti-bovine TSH-β, or nonimmune serum. The source of these antisera and their specificity for [125I]iodo-TSH and subunits prepared by chloramine T iodination (10) are described in Table I. Samples were incubated for 2½ h at 37°C after which antigen-antibody complexes were precipitated by the addition of 50 μl of staphylococcal Protein A suspensions (Calbiochem) according to the method of Kessler (12). In certain instances, the supernatant remaining after precipitation of antigen-antibody complexes was reprecipitated with either anti-bLH-β, or anti-bTSH-β.

Dissociation of TSH into Subunits—In certain instances, [35S]- or [3H]-labeled TSH was dissociated into free α and β subunits prior to immunoprecipitation by incubation of media or lysates in 0.1 M glycine, pH 2.4, at 37°C for 2 h. We and others (13) have previously demonstrated that such conditions result in > 95% dissociation of TSH into free α and β subunits that are intact by physicochemical and immunologic criteria as well as their ability to recombine quantitatively. After acid dissociation of TSH, incubates were immediately frozen. Samples were later thawed and titrated back to pH 7.4 by the
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addition of appropriate amounts of 4 M Tris base, pH 11.1. Immunoprecipitation was then performed immediately to prevent recombination of α and β subunits.

Analysis of Immunoprecipitated Products—Immune complexes were eluted from Staph A and reduced by incubation at 100°C for 2 min in a solution containing 1% (w/v) SDS, 5% (w/v) mercaptoethanol, 20% (w/v) sucrose, 0.1 M Tris-HCl, 0.02 M EDTA, and 0.1% (w/v) bromphenol blue. Samples were analyzed by electrophoresis in SDS-polyacrylamide slab gels (1.5 mm thick) with a 5% polyacrylamide stacking gel (1 cm) and a 12 to 20% linear gradient resolving gel (9 cm) as previously described (5). After electrophoresis, gels were fixed and stained with protein Coomassie brilliant blue (Eastman Organic Chemicals). The following protein standards (2 to 5 μg each obtained from Calbiochem or Sigma) were mixed with each sample immediately prior to gel electrophoresis to serve as internal molecular weight markers: albumin (68,000), immunoglobulin heavy chain (55,000), ovalbumin (43,000), chymotrypsinogen (25,000), immunoglobulin light chain (24,000), soybean trypsin inhibitor (20,000), myoglobin (17,000), and cytochrome c (12,000). Molecular weights were determined by the method of Lambin (14).

Stained gels were sliced into 1-mm sections, solubilized, and de- stained in 0.5 ml of 30% (v/v) hydrogen peroxide overnight at 50°C and counted in 10 ml of a toluene-based, detergent-containing counting solution (Ready-Solv HP, Beckman). Samples were counted on a Beckman liquid scintillation counter (LS-9000) after initial monitoring by the random coincidence detector indicated that residual chemiluminescence was negligible. Samples were also monitored for counting efficiency by use of an automatic external γ standard. For 35S, all samples displayed a uniform counting efficiency of 94% and these data are expressed as counts per min. For 3H, there was slight variability of counting efficiency among samples (41 to 46%) and, therefore, these data are expressed as disintegrations per min. All samples were counted for the time necessary to achieve less than 5% counting error in major peaks. Total radioactivity in various regions of the gel was calculated by the automatic integration program of the Beckman LS-9000. All data have been corrected for radioactive decay.

RESULTS

Pulse-Chase Labeling with [35S]Methionine—After a 10-min pulse of [35S]methionine and no chase (0 min), the intracellular forms immunoprecipitated by the anti-α serum were predominantly of apparent Mr = 18,000 with a second component Mr = 21,000, the latter corresponding to the size of "standard" pituitary TSH-α (Fig. 1A). There were also minor α components of Mr = 14,000 to 15,000, corresponding to the position of "pre-α," the product of cell-free translation, as well as other forms of Mr < 12,000. At this initial time, the anti-β serum precipitated mainly one form of Mr = 18,000, corresponding to the apparent size of standard TSH-β. Nonimmune

![Figure 2](image_url)

Fig. 2. [35S]methionine-labeled subunits synthesized during continuous labeling. Lysate radioactivity after 30 to 240 min of continuous exposure to [35S]methionine, compare with 10-min pulse in Fig. 1A. Medium radioactivity (not shown) was similar to that in pulse-chase study (Fig. 1C). Total volumes of lysate were 2 ml and volume immunoprecipitated at 30 and 60 min was 0.12 ml and at 120 and 240 min was 0.04 ml. To normalize total counts with respect to

| TABLE I |
|----------|
| Specificity of rabbit antisera in the precipitation of rat [125I]iodo-TSH and free subunits (see "Experimental Procedures") |
| Rat TSH and subunits are chemically and immunologically similar to mouse forms (10, 15) and are the only rodent materials available in pure form. Numbers represent percentage of radioactivity immunoprecipitated by each serum with or without the presence of experimental culture medium. |
| TSH | LH-α* | TSH-β* |
|------|-------|-------|
| Anti-LH-α* | 100  | 100  |
| Anti-TSH-β* | 85   | <1   | 160  |
| Nonimmune serum | <1   | <1   | <1   |

*Chemically identical with α prepared from TSH.

*Generated at National Institutes of Health by immunization with bovine LH-α, prepared by Dr. J. G. Pierce, University of California, Los Angeles.

A gift of Dr. I. A. Kourides, Memorial Sloan-Kettering Cancer Institute, who generated by immunization with bovine TSH-β prepared by Dr. J. G. Pierce.

Fig. 1, A and B, multiply 30- and 60-min counts per min by 2, and 120 and 240 min by 6. Symbols as in legend to Fig. 1.
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When the initial 10-min pulse was followed by chase periods of 10 to 240 min, the 18,000 α form was converted progressively into the 21,000 form (Fig. 1A, B), implying a precursor-product relationship between the two. There was no consistent change in the very small amounts of intracellular α components of < 12,000. During the chase period, most of the radioactivity precipitated by anti-TSH-β remained at \( M_\text{r} = 18,000 \), although a small component appeared at \( M_\text{r} = 21,000 \). As will be demonstrated below, this latter component is not actually a form of β subunit but represents a subunit combined with TSH-β in TSH. There was no significant precipitation of radioactivity by nonimmune serum at any time during the chase period.

In the media, heterogeneous radioactive forms specifically precipitated by anti-α and anti-β first appeared at 120 min (Fig. 1C). There were two major classes of radioactivity precipitated by each antiserum, 18,000 to 19,000 and 20,000 to 23,000. As will be described below, the large molecular weight components are predominantly secreted α (both free and combined with β), while the smaller molecular weight component is predominantly secreted β (virtually all combined with α).

Between the 10-min pulse/0-min chase and the 10-min pulse/10-min chase, there was a significant increase in total immunoprecipitated radioactivity (Fig. 1A), presumably caused by a delay in equilibration to the final low specific activity of methionine pools as well as run-off of previously initiated labeled chains. However, between 10 to 240 min of chase, the sum of intracellular + extracellular immunoprecipitated radioactivity was relatively constant indicating no further \textit{de novo} synthesis or significant degradation of \([{}^{35}S]\)methionine-labeled subunits.

Continuous Labeling with \([{}^{35}S]\)Methionine—Continuous labeling experiments of 30 to 240 min showed greater amounts of the intracellular form \( M_\text{r} = 21,000 \) of α compared to the form \( M_\text{r} = 18,000 \) at all periods (Fig. 2A), in contrast to the 10-min pulse (Fig. 1A). However, in contrast to the pulse-chase labeling, in continuous labeling the ratio of the two intracellular α forms remained relatively constant, presumably because of continuing new synthesis of the smaller form and selective secretion of the larger form. The predominant intracellular β form was also of \( M_\text{r} = 18,000 \). However, as in pulse-chase labeling, from 60 to 240 min there was the appearance of a second peak \( M_\text{r} = 21,000 \) precipitated by anti-β that represents the α subunit within TSH (see below). The media of continuous labeling experiments displayed a pattern of immunoreactive material, first appearing at 120 min, similar to that of the pulse-chase studies (data not shown; see Fig. 1C).

**Determination of Total and Free Subunits**—Our previous biosynthesis studies using gel chromatography under non-denaturing conditions had indicated that α and β subunit combination to form complete TSH began within 60 min of the chase period (7). The presence of such complete TSH could lead to ambiguity in the identification of α and β subunits by the current method of immunoprecipitation and SDS-gel analysis, since both anti-α and anti-β precipitate complete TSH (Table I). Such TSH precipitated by either antiserum subsequently dissociates into both α and β subunits during the reduction and denaturation of SDS-gel electrophoresis. Moreover, it was not possible to devise conditions of electrophoresis combined forms ("total subunits"). Lysate (2nd from top) and media (bottom panel) were also immunoprecipitated (without prior acid exposure) with one antiserum and the supernatant remaining was then precipitated with the other antiserum to yield "free" subunits (see "Experimental Procedures" and "Results").
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(i.e. elimination of reduction step) that would preserve \( \alpha-\beta \) combination in TSH while allowing complete dissociation of the antibody-TSH complex.

In order to clarify the identification of \( \alpha \) and \( \beta \) after subunit combination had occurred, labeled TSH in lysate and media at 240 min was acid-dissociated prior to immunoprecipitation (see "Experimental Procedures"). Under these conditions, the radioactivity precipitated by each antiserum was specific for one subunit (see Table I) and represented both free and previously combined forms ("total subunits"). In both the lysate and medium, prior acid dissociation eliminated most of the precipitation of the 21,000-dalton form by anti-\( \beta \), identifying this form as \( \alpha \) within TSH (Fig. 3; compare with 240 min in Figs. 1 and 2). Acid treatment of medium eliminated all precipitation of the 18,000 form by anti-\( \alpha \), identifying this form as \( \alpha \) within TSH. In the lysate, acid dissociation eliminated only part of the 18,000-dalton form precipitated by anti-\( \alpha \) (i.e. \( \beta \) in TSH); the remainder represented the small \( \alpha \) form.

In order to identify directly subunits that were not combined within TSH ("free subunits"), the supernatant remaining after precipitation with one antiserum (without prior acid dissociation) was then precipitated with the other antiserum. For example, anti-\( \alpha \) would precipitate free \( \alpha \) as well as \( \alpha \) and \( \beta \) combined within TSH, leaving free \( \beta \) (and other non-TSH radioactivity) in the supernatant, which was then precipitated with anti-\( \beta \). Using these methods, it was demonstrated for the lysate at 240 min that, of the total subunits identified above, about 50% of the 21,000-dalton \( \alpha \) form and 50% of the 18,000 \( \beta \) form were "free" while virtually all of the 18,000 \( \alpha \) form was free. In the medium, about 50% of the exclusive 23,000-dalton \( \alpha \) form was free, while no free \( \beta \) was detected.

**Ratio of \( \alpha/\beta \) Subunits**—The prior acid dissociation study permitted an unequivocal identification of all molecular weight forms of \( \alpha \) and \( \beta \) subunits and, therefore, a reliable estimate of the \( \alpha/\beta \) ratio at 240 min of continuous labeling. The \([\text{S}]\)methionine \( \alpha/\beta \) ratio of the lysate was 5.2, that of the medium was 3.8, and the total ratio was 4.0. Similar ratios were estimated at other times of the pulse-chase study, taking into account the proportion of each subunit precipitated by each antiserum (see above).

In order to calculate the molar \( \alpha/\beta \) ratio, knowledge of the methionine content of each subunit is necessary. This is not

### Table I

**Relative incorporation of \([\text{S}]\)methionine or \(^3\text{H}\)-carbohydrates into \( \alpha \) and \( \beta \)**

|          | Methionine | Mannose | Glucosamine | Galactose | Fucose |
|----------|------------|---------|-------------|-----------|-------|
| TSH-\( \alpha \) |             |         |             |           |       |
| Peak I    | 1,260      | 121     | 385         | 122       | 281   |
| Peak II   | 830        | 78      | 178         | 64        | 254   |
| I/II      | 1.52       | 1.55    | 2.17        | 1.90      | 1.11  |
| (I/II) normal-ized  | 1.0       | 1.4     | 1.3         | 0.73      |       |
| TSH-\( \beta \) |             |         |             |           |       |
| Peak I    | 311        | 12      | 122         | 39        | 56    |
| Peak II   | 1,451      | 47      | 326         | 429       |       |
| I/II      | 0.214      | 0.255   | 0.374       | 0.274     | 0.131 |
| (I/II) normal-ized  | (1.2)     | 1.7     | (1.3)       | 0.61      |       |

a Total radioactivity (dpm/ml) with \( M_i = 20,000-23,000 \).

b Total radioactivity (dpm/ml) with \( M_i = 16,000-19,000 \).

c (I/II) labeled carbohydrate + (I/II) labeled methionine.

d Ratios unreliable because of sample counting error >10%.

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**Fig. 4. Incorporation of \(^3\text{H}\)-carbohydrates.** Lysate radioactivity after 120 min of continuous exposure to either \([\text{S}]\)methionine (top panel), \([\text{H}]\)fucose (2nd from top), \([\text{H}]\)glucosamine (3rd from top), \([\text{H}]\)galactose (4th from top), or \([\text{H}]\)mannose (bottom). All samples were acid-dissociated prior to immunoprecipitation to yield total subunits (see legend to Fig. 3 and Table I).
known for mouse TSH subunits, although the methionine content of mouse pituitary LH-α (presumably identical with TSH-α) is known to be 3 residues and that of mouse LH-β is 2 (15). Interestingly, a similar value of approximately 3:2 is derived from the ratio of radioactivity in the 21,000-dalton peak to that of the 18,000-dalton peak precipitated (without prior acid dissociation) in the medium by anti-TSH-β (Fig. 1C and Fig. 5). Since the acid-dissociation studies have identified these two peaks as combined α and β, respectively, and since there is no free β in the media (see above), the two peaks should contain equimolar amounts of α and β. Therefore, the ratio of methionine residues in mouse TSH-α and TSH-β may also be approximately 3:2, and the molar α/β ratios are likely to be 2:3 of the [35S]methionine ratios or 2.7 for the total ratio.

Incorporation of 3H-Carbohydrates—In a separate experiment, the incorporation of [35S]methionine was compared to that of various 3H-carbohydrates (Fig. 4, Table II). Intracellular radioactivity was precipitated after acid dissociation of 120 min of constant labeling in order to determine the relative carbohydrate content of the two intracellular α forms that had been recognized by [35S]methionine labeling. Both forms of α were labeled with fucose, glucosamine, galactose, and

![GEL SLICE (mm)](image)

**Fig. 5.** Effect of tunicamycin. Media after 24 h continuous exposure to tunicamycin and labeled precursors added simultaneously at zero time were immunoprecipitated without prior acid dissociation. A, [35S]methionine: top panel, no tunicamycin; middle, tunicamycin, 1 μg/ml; bottom, 5 μg/ml. B, [3H]glucosamine: top panel, no tunicamycin; bottom, 5 μg/ml. At 5 μg/ml, tunicamycin caused an 83% inhibition of total [35S]-labeled α synthesized (see "Results").
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mannotose. There was, however, no significant incorporation of N-acetylmannotose, a specific precursor of sialic acid (data not shown), consistent with the known absence of sialic acid in mouse and other subprimate TSH (15).

In addition to the expected heterogeneous α forms, labeling with carbazole identified an unexpected higher molecular β form of M, = 21,000 that was not clearly shown by labeled methionine. This form of β was most evident after glucosamine labeling (Fig. 4).

The relative incorporation of various 3H-carbohydrates into high and low molecular weight subunit forms is examined quantitatively in Table II. Using [35S]methionine incorporation to normalize for the relative amounts of polypeptide in various forms, we noted that, compared to the lower molecular weight form, the higher molecular weight form of α incorporated relatively more labeled glucosamine and less fucose. The incorporation of the latter carbohydrate was particularly high in the small molecular weight β (M, = 18,000, corresponding to standard pituitary TSH-β, see Fig. 4), in agreement with studies showing a relatively high fucose content of standard β (15).

Effect of Tunicamycin.—Various amounts of tunicamycin, an inhibitor of the formation of N-acetylglicosaminolipid intermediates necessary for glycosylation at asparagine residues (16), were added to cultures throughout a 24-h incubation period with [35S]methionine or [3H]glucosamine (Fig. 5). The addition of 1 µg/ml caused the appearance in the media of a new [35S]methionine peak M, = 11,000 to 13,000 that was precipitated selectively by the anti-α serum. At 5 µg/ml, the predominant [3S]-labeled α form was M, = 11,000. A very small amount of anti-β-precipitable radioactivity in the range M, = 11,000 to 13,000, appeared in the media after tunicamycin treatment. This could be a β form or could represent a small amount of the 11,000-dalton α form combined within TSH. However, most of this α form was free.

No [3H]glucosamine was detected in the M, = 11,000 α form, even at a tunicamycin dose of 5 µg/ml. This directly confirmed the fact that glycosylation was fully inhibited in this form. Moreover, the molecular weight of this form agrees with that of the α protein core which is also approximately 11,000 (17).

At the doses of tunicamycin necessary to achieve major inhibition of glycosylation, there was also some inhibition of total [35S]methionine incorporation into both α and β. At 1 µg/ml incorporation into subunits was inhibited about 64%, and at 5 µg/ml, 83%. Preincubation of cells with tunicamycin for 16 h before addition of labeled methionine or glucosamine more effectively inhibited glycosylation of labeled α, but caused a greater inhibition of labeled α synthesis (data not shown). Although tunicamycin caused a significant inhibition of total (lysat + media) [35S]methionine-labeled subunits, it did not cause a major change in the ratio of media to total (or media to lysate) subunits. Thus, in cultures with 0, 1, and 5 µg/ml of tunicamycin, greater than 90% of total [3S]-subunits were recovered in the media after 24 h of continuous labeling.

However, we have not yet examined the possibility that tunicamycin treatment resulted in a retention and rapid intracellular destruction of certain nonglycosylated forms.

After 24 h of continuous labeling of media with or without tunicamycin, it was noted that the largest molecular weight peak precipitated by anti-α (M, = 22,000 to 23,000) was slightly but reproducibly different from the largest molecular weight peaks precipitated by anti-β (M, = 20,000 to 21,000) (Fig. 5). Since we had demonstrated that the former represents both free and combined a while the latter represents solely combined α, these data suggest that secreted free α may be more glycosylated than secreted a combined with β (see “Discussion”). A similar finding had been noted in media labeled at earlier intervals (Fig. 1C), but not in intracellular forms at any time interval (Fig. 1, A to B, 2), suggesting that extracellular free α was more glycosylated than intracellular free α.

**DISCUSSION**

Pituitary TSH purified from various species has a M, = 28,000 and contains approximately 20% carbohydrate (15). Only bovine, porcine, and human TSH have been characterized in detail, including the amino acid sequence and carbohydrate composition of each subunit (15, 17, 18). For bovine TSH, the α subunit has a M, = 13,600, of which 10,800 is comprised of a protein core of 96 amino acid residues and 2,800 (21%) represents two oligosaccharide units linked to asparagine residues via N-glycosidic bonds. Bovine TSH-β has a M, = 14,700, of which 13,100 is comprised of a protein core of 113 amino acid residues and 1,600 (12%) represents one asparagine-linked oligosaccharide unit. As is true for other glycoproteins of similar carbohydrate composition, TSH subunits migrate anomalously in SDS-gel electrophoresis, α with an apparent M, = 21,000, and β with an apparent M, = 18,000 (see “Results”).

Studies of TSH biosynthesis by translation of messenger RNA in cell-free systems have demonstrated that the α and β subunits are synthesized from separate mRNA as “pre-subunit” forms (2-6). Pre-TSH-α has an apparent M, = 14,000 to 17,000 (depending on the conditions of gel electrophoresis (5)) similar to that of pre-hCG-α, which had been described earlier (19, 20). We have recently demonstrated that pre-α translated from mouse pituitary tumor mRNA contains a hydrophobic NH-term (“signal peptide”) of about 3,000 daltons (5). This peptide displayed amino acid sequence homology with normal mouse and bovine pituitary pre-α as well as human placental pre-α (20). Pre-TSH-β has an apparent M, = 15,500 and, although not yet chemically characterized, presumably also contains a signal peptide since it is 3,000 daltons larger than nonglycosylated TSH-β (6).

TSH subunits have been “processed” (glycosylated and cleaved of the signal peptide) either by translation of mRNA in intact frog oocytes (3) or by addition of crude microsomal membranes during translation in cell-free systems (6). However, studies in intact TSH-producing cells are necessary to define the physiologic mechanisms of post-translational processing including proteolytic cleavage of precursors, glycosylation, and subunit combination as well as TSH and subunit secretion.

Intact cell biosynthetic studies from our laboratory (7) were initially performed using primary monolayer cultures of mouse pituitary tumor cells which we had shown to synthesize primarily TSH and its free α subunit, but no gonadotropin, prolactin, or growth hormone (5, 10). These studies indicated that the earliest immunoactive α form identified intracellularly during a 10-min pulse with [35S]methionine was smaller than standard α by gel chromatography and was converted to higher molecular weight α forms during a 60-min chase period. Free β subunit was not clearly identified, but complete TSH appeared in cells by 60 min of the chase period; TSH and excess free α appeared in the media by 120 min.

In the current studies, we employed primary cultures of thyrotropic tumor cells in suspension after overnight preincubation rather than the previous monolayer cultures confluent after 6 days, since the latter have been shown to contain predominantly fibroblasts (21), leading to nonspecific precipitation of high molecular weight collagen components (7).
Furthermore, we employed the Staph A immunoprecipitation rather than the previous double antibody method and analyzed the eluted immune complexes by SDS-gradient polyacrylamide gel electrophoresis after reduction and denaturation. These methodologic improvements virtually eliminated nonspecific precipitation of radioactivity and permitted high resolution analysis of various forms of α and β subunits.

The initial intracellular α forms identified after the 10-min pulse were predominantly apparent \( M_s = 18,000 \), with a second component of \( M_s = 21,000 \), the latter corresponding to standard α. Although we have not yet demonstrated that this initial \( M_s = 21,000 \) form was derived from a \( M_s = 18,000 \) form, such precursor-product relationships between 18,000 and 21,000 forms were demonstrated at later times during the chase period. At 120 min, we demonstrated that, compared to the \( M_s = 18,000 \) form, the \( M_s = 21,000 \) α form incorporated more labeled glucosamine and galactose, but similar amounts of mannose and less fucose. These data suggest that, at least at 120 min, the two forms differ primarily in outer residues of glucosamine, galactose, and fucose which are added after translation, rather than in core residues of glucosamine and mannose, which are added to the nascent polypeptide chain by oligosaccharide-lipid intermediates (see below). In the medium, none of the \( M_s = 18,000 \) α form was detected.

Small amounts of α forms \( M_s < 12,000 \) were noted only intracellularly, but these were variable among experiments and showed no pulse-chase relationships with other forms. In other experiments not shown, prolonged incubation of cells lysates with crude intracellular membranes generated more of these forms, suggesting that they were the products of proteolysis. However, such proteolysis was usually minimal since virtually all of the total α was conserved during the pulse-chase study (Fig. 1). During the 10-min pulse, a very small α peak \( M_s = 14,000 \) to 16,000 was noted in the region corresponding to pre-α (Fig. 1A). However, in the intact cell, most of the signal peptides should be cleaved even before completion of nascent chains (22), and preproteins are very difficult to demonstrate even with much shorter pulse periods (1/2 to 2 min) as well as the use of proteolytic enzyme inhibitors (23-25).

Only one form of \( [\text{S}]\text{methionine-labeled} \) β, \( M_s = 18,000 \) corresponding to standard pituitary TSH-β, was identified either intracellularly or extracellularly. However, using \( [\text{H}] \) carbohydrates, a higher molecular weight intracellular form \( M_s = 21,000 \) was also noted (Fig. 4) and incorporated more labeled glucosamine and less fucose (Table II).

By comparing the pattern of immunoprecipitation with and without prior acid dissociation of combined α and β subunits, it was possible to deduce that subunit combination (as judged by precipitation of \( M_s = 21,000 \) \(^{35}\)S radioactivity by anti-β) began as early as 10 to 30 min of the chase period (see “Results” and Fig. 1). Moreover, by precipitation of supernatants remaining after prior immunoprecipitation, it was possible to demonstrate directly subunits that were uncombined (see “Results” and Fig. 3). Free α \( M_s = 18,000 \) and 21,000 as well as free β \( M_s = 18,000 \) were found in cells but only free α \( M_s = 22,000 \) was found in the medium. The absence of free β in the media is consistent with prior studies suggesting that the β subunit is limiting in glycoprotein hormone biosynthesis. Moreover, free α in the media appeared to be slightly larger, and presumably more glycosylated, than media α combined with β in TSH or than any form of intracellular α.

The present studies confirm data from cell-free studies (2-6), showing that α and β subunits are synthesized from separate mRNA without a larger common precursor form that contains both chains, as in proinsulin (26). Nor is there evidence for any larger pro-α or pro-β forms such as proparathyroid hormone (27) or pro-corticotropin-endorphin (28), which should be readily identifiable for at least 30 to 60 min of pulse-chase (26-28). It is possible that a very small additional propeptide piece (i.e. < 10 amino acid, including certain tryptophan residues) present during the biosynthesis of either subunit escaped detection in this study. Although NH2-terminal microsequencing of pre-hCG-α (29) and pre-TSH-α (5) shows no evidence for such a propeptide, it might be present at the COOH terminus, since hCG-β contains an uncleaved COOH-terminal extension of 40 residues (29). However, it seems more probable that, like growth hormone, prolactin, and placent lactogen (22, 24, 30, 31), glycoprotein hormone subunits are not synthesized via propeptides.

As in previous studies of TSH and other glycoprotein hormone biosynthesis, we found excess production of α compared to β subunit (2-6, 10, 19). We have shown previously that the degree of unbalanced α production is greater in pituitary tumors compared to normal pituitaries and may vary even among mouse pituitary thyrotropic tumors (10). In the current biosynthetic studies, the \( [\text{S}]\text{methionine} \) α/β ratio was approximately 4.0 and the molar α/β ratio was estimated to be 2.7 (see “Results”). The significance of increased α/β production is not known, but presumably would allow regulation of hormone biosynthesis by the limiting β subunit while assuring its rapid combination with the excess α (7, 32, 33). Increased light chain compared to heavy chain production has also been noted in immunglobulin-producing cells (34).

Tunicamycin is an antibiotic that has been shown to specifically inhibit formation of \( N\)-acetylgalosaminylyphosphorylpolysorofrenol (16) which is involved in the synthesis of the core oligosaccharides linked to asparagine. Since only such \( N\)-glycosidically linked oligosaccharides are present in TSH (15), it was expected that this drug could totally block subunit glycosylation. After exposure to 5 μg/ml of tunicamycin, the predominant \( [\text{S}]\text{methionine} \) α form in the medium was \( M_s = 11,000 \) to 12,000 and contained no \( [\text{H}]\)glucosamine, consistent with a completely nonglycosylated protein. Nonglycosylated β was suggested but not clearly identified in the expected region \( M_s = 13,000 \). Although tunicamycin caused a slight inhibition of α secretion, the large amounts of nonglycosylated α in the media is consistent with other reports showing that glycosylation of glycoproteins is not necessarily a prerequisite for secretion (35, 36).

Neither the nonglycosylated α produced by tunicamycin nor the \( M_s = 18,000 \) intracellular form which is presumably only partially glycosylated, was found combined with β subunit in complete TSH. These data suggest that adequate glycosylation may be necessary for α-β subunit combination, although direct cell-free subunit combination studies will be necessary to test this hypothesis. Interestingly, such direct studies have been performed with secreted free forms of hCG-α which, like the secreted free form of TSH-α in the current studies, appear to be more glycosylated than standard α dissociated from complete hormones (33, 37). These free forms showed diminished combining activity compared to standard α, suggesting that overglycosylation, as well as underglycosylation, can inhibit subunit combination.

Recent studies of hCG biosynthesis by choriocarcinoma cells also reveal a small intracellular α precursor of \( M_s = 18,000 \) as well as selective secretion of a molecular weight α form \( M_s = 22,000 \), corresponding to standard hCG-α (38, 39). As in the present studies, there was evidence that the secreted free α subunit was more glycosylated than the secreted α combined with β (39). However, unlike the present studies, none of the hCG-α \( M_s = 22,000 \) accumulated in cells, perhaps related to an observed faster rate of secretion of hCG and excess free α.
It will be important to characterize the carbohydrate composition and structure of the various intracellular and extracellular subunits by direct chemical methods. It is likely that individual SDS-gel peaks are themselves heterogeneous, and other physicochemical methods may be required for better resolution as well as for more reliable estimation of molecular weight. The H-carbohydrates used in the current studies were probably not metabolized appreciably to other carbohydrates (40, 41), the relatively low incorporation prevented chemical characterization of the H-labeled products.

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