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Temporal Pattern of Incorporation of ^3H Precursors into Pituitary Glycoproteins and Their Subsequent Release (41374)

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Abstract. The temporal pattern of incorporation of various ^3H precursors into glycoproteins by rat anterior pituitaries incubated *in vitro* and the release of ^3H-glycoproteins was examined. [^3H]Leucine incorporation was linear with respect to time and [^3H]leucine-containing macromolecules appeared in the media in about 1 hr. The temporal pattern of [^3H]mannose incorporation and release was similar to that observed for [^3H]leucine consistent with [^3H]mannose incorporation via the chitobiose core. [^3H]Galactose and [^3H]fucose were incorporated after apparent time delays of approximately 15 min and soon thereafter (20–25 min) appeared in the medium in ^3H-glycoproteins. Thus, these precursors appear to be added as terminal residues. [^3H]Glucosamine exhibited a pattern intermediate between [^3H]leucine and [^3H]fucose whereas [^3H]GlcNAc appeared to be incorporated as a terminal residue.

The three anterior pituitary glycoprotein hormones LH (luteinizing hormone, Lutropin),[1] FSH (follicle-stimulating hormone, Follitropin), and TSH (thyroid-stimulating hormone, Thyrotropin) contain complex, asparagine-linked carbohydrate moieties (1). The biosynthesis of these hormones as well as general adenohypophysial glycoprotein biosynthesis remains to be more fully investigated. In a variety of tissues, the biosynthesis of glycoproteins containing complex, asparagine-linked carbohydrate residues involves protein synthesis on bound ribosomes, synthesis of the internal carbohydrate core on the isoprenol lipid dolichol pyrophosphate, transfer of the carbohydrate core to the nascent protein chain, trimming of excess carbohydrate residues by enzymes on the inner surface of the endoplasmic reticulum, and addition of peripheral carbohydrate residues by glycosyltransferases located in the Golgi apparatus (reviewed in (2)). Some, but not all, of these steps have been demonstrated in adenohypophyseal tissue. Godine *et al.* (3) observed that rat LH and FSH are synthesized from presubunit molecules. Henner *et al.* (4) have shown that the bovine pituitary synthesizes the internal carbohydrate core consisting of two GlcNAc, nine mannose, and one to three glucose residues on dolichol pyrophosphate. Landefeld and Kepa (5) have demonstrated that the subunits of bovine LH are glycosylated while the peptide chain is nascent on the ribosome. These studies (3–5) have used cell-free systems. Other steps in general or specific glycoprotein biosynthesis by the anterior pituitary remain to be demonstrated.

In the present investigation, the temporal pattern of incorporation of ^3H precursors into glycoproteins and their subsequent release by rat anterior pituitaries incubated *in vitro* was characterized. By employing this intact tissue system, the net result of all the processes involved in glycoprotein biosynthesis can be examined.

Materials and Methods. *Anterior pituitary incubations.* Mature, male, Long Evans rats were killed by decapitation.

[1] Abbreviations used: LH, luteinizing hormone, Lutropin; FSH, follicle-stimulating hormone, Follitropin; TSH, thyroid-stimulating hormone, Thyrotropin; KRB-MEM, Krebs–Ringer's bicarbonate buffer containing 10 mmole/liter glucose and minimum essential medium amino acid and vitamin mixtures; TCA, trichloracetic acid; PPO, 2,5-diphenyloxazole; DMPOPOP, 1,4-bis[2-(4-methyl-*s*-phenyloxazolyl)]-benzene; GlcNAc, *N*-acetyl-D-glucosamine.

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Their anterior pituitaries were removed, hemisectioned, and preincubated. The preincubation medium (1 ml/gland) consisted of Krebs–Ringer's bicarbonate buffer (118.5 mmol/liter NaCl, 4.7 mmol/liter KCl, 2.5 mmol/liter $CaCl_2$, 1.2 mmol/liter KH_2PO_4, 1.2 mmol/liter $MgSO_4$, 24.9 mmol/liter $NaHCO_3$) supplemented with 10 mmol/liter glucose, and Eagle's minimum essential minimum amino acid and vitamin mixtures (Gibco, Grand Island, N.Y.) (KRB-MEM). The shaking water bath incubator was maintained at 37° and 95% O_2/5% CO_2 was used as the gas phase. After preincubation for 2 hr, two hemipituitaries were weighed and incubated in 0.5 ml KRB-MEM for various intervals in the presence of one of the ^3H precursors listed in Table I. ^3H Precursors were purchased from New England Nuclear (Boston, Mass.) or Amersham (Arlington Heights, Ill.) and were added at a concentration of 10 μCi/ml. The specific activities of particular precursors are presented in relevant figures. When the precursor was [^3H]leucine, the MEM amino acid mixture was omitted from the incubation medium. In one experiment, glucose deprivation was achieved by substituting 10 mmol/liter succinate for glucose in the KRB-MEM (6).

The incubation was terminated by adding 0.5 ml KRB-MEM containing 2 mg/ml of unlabeled precursor corresponding to the ^3H compound being examined. The tissue was removed from the incubation medium, rinsed with KRB-MEM (0.2 ml/gland), and homogenized in distilled water supplemented with 1 mg/ml of unlabeled precursor corresponding to the ^3H compound under consideration. ^3H-Glycoproteins in aliquots of the incubation medium and tissue homogenate were precipitated with trichloroacetic acid (TCA). Release was defined as the TCA-precipitable ^3H-glycoproteins in the incubation medium whereas incorporation was defined as the TCA-precipitable ^3H-glycoproteins in the tissue homogenate plus those in the incubation medium. For some precursors, uptake and acid-soluble pools were also quantitated. Uptake was defined as the quantity of ^3H precursor in the tissue homogenate plus the TCA-precipitable ^3H-glycoproteins in the incubation medium. The acid soluble pool was the quantity of ^3H precursor in the tissue homogenate not TCA precipitable.

Precipitation of glycoproteins. Aliquots of incubation medium were substituted with 250 μg bovine γ-globulin to aid in the recovery of precipitates. These aliquots as well as aliquots of tissue homogenates were brought to 10% (w/v) TCA by adding an equal volume of cold (4°) 20% (w/v) TCA. After incubation in an ice bath for 15 min, precipitates were recovered by centrifugation (1500*g*, 15 min). They were washed twice with cold 10% TCA, once with acetone: 0.2 *M* NaCl (9:1, v/v), twice with ethanol:ether (2:1, v/v), and once with ether. After each wash, precipitates were recovered by centrifugation (1500*g*, 15 min). Residual ether was removed by placing the samples in a 60° water bath. Precipitates were dissolved in 1 *N* NaOH, transferred to scintillation vials, neutralized, and counted in a toluene:Triton X-100 (2:1, v/v) scintillation fluid containing 5 g/liter 2,5-diphenyloxazole (PPO) and 0.1 g/liter 1,4-bis[2-(4-methyl-s-phenyloxazolyl)]-benzene (DMPOPOP). Aliquots of tissue homogenates and ^3H precursors in tissue homogenates not TCA precipitable were dissolved in NCS tissue solubilizer (Amersham, Arlington Heights, Ill.) neutralized with acetic acid and counted in a toluene-based scintillation fluid containing 5 g/liter PPO and 0.1 g/liter DMPOPOP. Quench correction was achieved by the channels-ratio method. Results were expressed as disintegrations per minute per microgram tissue protein.

For each precursor, time zero control samples were utilized to assess nonspecific precipitation. KRB-MEM containing 2 mg/ml of unlabeled precursor was added prior to adding the tissue or the ^3H-glycoprotein precursor. For each ^3H precursor, nonspecific precipitation was near the background of the liquid scintillation center.

Incorporation and release rates were described by linear regression lines calculated by standard least-squares techniques. Apparent delays until incorporation or release were taken as the x intercepts of the regres-

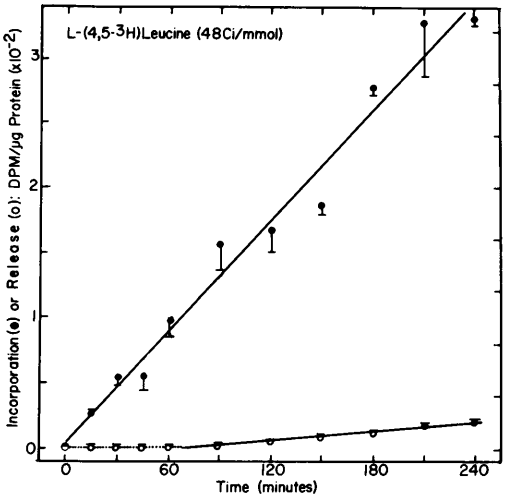

FIG. 1. The temporal pattern of incorporation of L-[4,5-^{3}H]leucine into proteins and glycoproteins and the release of ^{3}H macromolecules by rat anterior pituitaries incubated *in vitro*. Each point represents the mean ± SD of triplicate observations.

sion lines. When calculating release rates, points between 60 and 240 min were always utilized; earlier time points were excluded if they decreased the correlation coefficient.

Protein. The protein concentrations of tissue homogenates were determined by the method of Lowry *et al.* (7) using bovine serum albumin as the standard.

Results and Discussion. Rat anterior pituitaries incubated *in vitro* incorporated [^{3}H]leucine into macromolecular material linearly with respect to time (Fig. 1). [^{3}H]Leucine-containing proteins and glycoproteins appeared in the media approximately 1 hr after the addition of [^{3}H]leucine (Fig. 1, Table I). Glycoprotein precursors incorporated into complex-type glycoproteins via a chitobiose core mechanism should be released (appear in the incubation medium) with a time delay approximately equal to that observed for [^{3}H]leucine. Of the precursors examined, [^{3}H]mannose, [^{3}H]glucosamine, and [^{3}H]GlcNAc could potentially be incorporated in this manner. [^{3}H]Mannose incorporation was linear with respect to time and [^{3}H]mannose-containing glycoproteins appeared in the media approximately 1 hr after its addition (Fig. 2, Table I). These data are consistent with [^{3}H]mannose incorporated into pituitary glycoproteins via this mechanism. The uptake of [^{3}H]mannose was fairly rapid during the first hour and the rate subsequently declined. A similar pattern was observed by Todd and Samli (6).

In contrast to residues incorporated as a portion of the chitobiose core, residues may be added to glycoproteins by glycosyltransferases located in the Golgi apparatus. [^{3}H]Glucosamine could be incorporated as a portion of the inner carbohydrate core, as a peripheral residue or both. There was a slight time delay until [^{3}H]glucosamine incorporation (Fig. 3, Table I) which may represent the time required to enter the intracellular glucosamine pool and undergo *N*-acetylation. Nonetheless, [^{3}H]glucosamine-containing glycoproteins appeared in the media fairly rapidly. This precursor frequently exhibits a temporal incorporation and secretion pattern intermediate between core and peripheral residues (see (8)). Such appears to be the case in the present study. A curvilinear pattern of [^{3}H]glucosamine uptake was observed (Fig. 3). In contrast, Todd and Samli (6) observed a linear pat-

TABLE I. GLYCOPROTEIN BIOSYNTHESIS BY RAT ANTERIOR PITUITARIES INCUBATED *IN VITRO*

Precursor	Fig.	Delay until incorporation (min)	Delay until release (min)	Percentage released at 4 hr
L-[4,5-^{3}H]Leucine	1	None	66	5.8
D-[2-^{3}H]Mannose	2	None	56	4.2
D-[6-^{3}H]Glucosamine	3	22	None	5.9
N-Acetyl-D-[1-^{3}H]glucosamine	4	17	None	10.2
N-[^{3}H]Acetyl-D-glucosamine	5	None	3	38.0
D-[6-^{3}H]Galactose	6	15	24	2.3
L-[6-^{3}H]Fucose	7	15	23	12.0

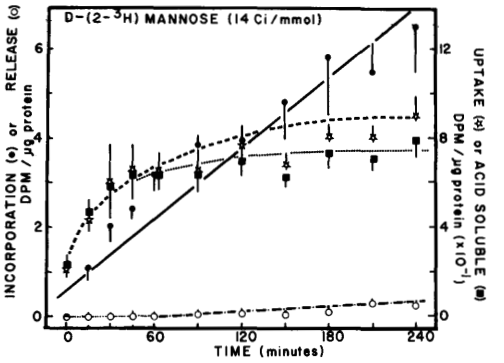

FIG. 2. The temporal pattern of uptake, incorporation of D-[2-^{3}H]mannose into glycoproteins, and the release of ^{3}H-glycoproteins by rat anterior pituitaries incubated *in vitro*. The acid-soluble [^{3}H]mannose pool is also illustrated. Each point represents the mean ± SD of triplicate observations.

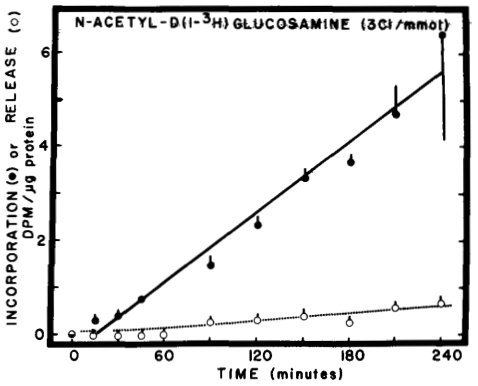

FIG. 4. The temporal pattern of incorporation of *N*-acetyl-D-[1-^{3}H]glucosamine into glycoproteins and the release of ^{3}H-glycoproteins by rat anterior pituitaries incubated *in vitro*. Each point illustrates the mean ± SD of triplicate observations.

tern of [^{3}H]glucosamine uptake. Although [^{3}H]GlcNAc could also be incorporated as a portion of the inner core, peripherally or both, there were minimal temporal delays until [^{3}H]NAcGlc incorporation and release (Figs. 4 and 5, Table I) suggesting incorporation as a terminal residue. [^{3}H]GlcNAc-containing glycoproteins appeared in the media soon after its addition.

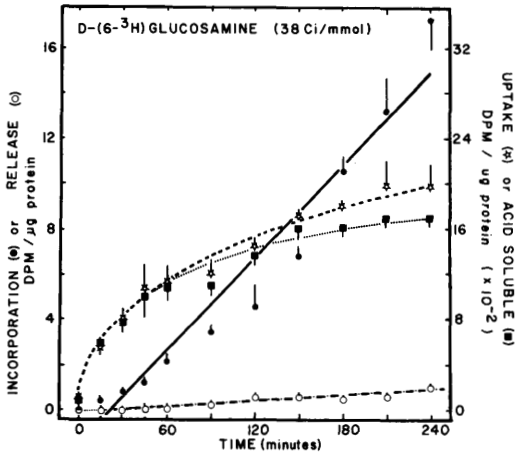

FIG. 3. The temporal pattern of uptake and incorporation of D-[6-^{3}H]glucosamine into glycoproteins and the release of ^{3}H-glycoproteins by rat anterior pituitaries incubated *in vitro*. The acid-soluble [^{3}H]glucosamine pool is also illustrated. Each point represents the mean ± SD of triplicate observations.

Galactose is a peripheral residue of glycoproteins (8). The incorporation of [^{3}H]galactose was linear with respect to time after an apparent delay of approximately 15 min (Fig. 6, Table I). [^{3}H]Galactose uptake was nearly linear during the first hour and the rate declined only slightly thereafter (Fig. 6). [^{3}H]Fucose, another precursor normally incorporated as a terminal residue was also incorporated after a delay of approximately 15 min (Fig. 7, Table I). Both [^{3}H]galactose- and [^{3}H]fucose-containing glycoproteins appeared in the media 20–25 min after the addition of the respective ^{3}H precursor. These data are consistent with [^{3}H]galactose and [^{3}H]Fucose incorporation as peripheral residues.

For each precursor, the percentage of the incorporated ^{3}H-glycoprotein precursor present in the incubation medium at 4 hr was calculated using the least-squares regression lines (Table I). This value would be affected by at least two factors: (a) residues incorporated in peripheral positions should appear in the media quicker than those incorporated via the chitobiose core and (b) residues incorporated into exportable glycoproteins would appear in the incubation medium whereas those incorporated into membrane or intracellular glycoproteins would not. Four to six percent of the [^{3}H]leucine-, [^{3}H]mannose-, and [^{3}H]-

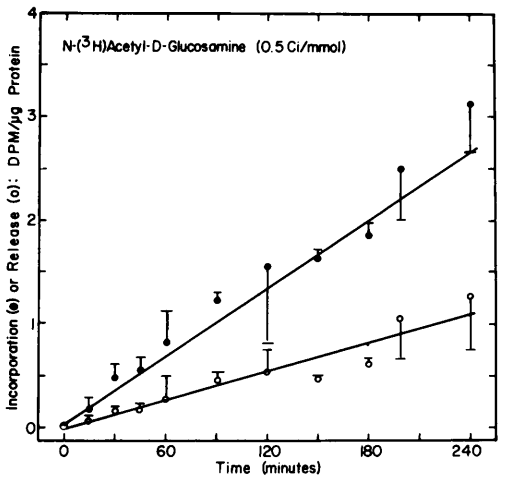

FIG. 5. The temporal pattern of incorporation of *N*-[^{3}H]acetyl-D-glucosamine into glycoproteins and the release of ^{3}H-glycoproteins by rat anterior pituitaries incubated *in vitro*. Each point represents the mean ± SD of triplicate observations. In this experiment, 10 m*M* succinate was substituted for glucose in the incubation medium.

glucosamine-containing glycoproteins were released at 4 hr (Table I). These values are quite comparable to the values reported by Todd and Samli (6). A considerably higher percentage of [^{3}H]GlcNAc-containing glycoproteins were secreted at 4 hr (Table I) consistent with incorporation late in the

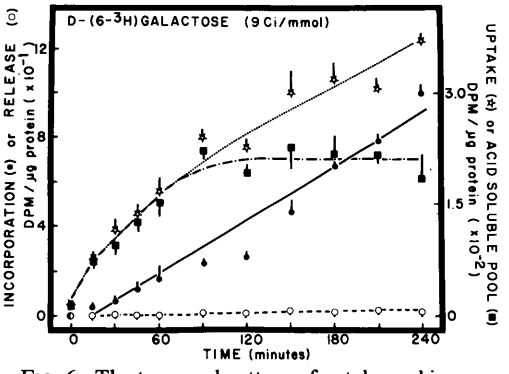

FIG. 6. The temporal pattern of uptake and incorporation of D-[6-^{3}H]galactose into glycoproteins and the release of ^{3}H-glycoproteins by rat anterior pituitaries incubated *in vitro*. The acid-soluble [^{3}H]galactose pool is also illustrated. Each point represents the mean ± SD of triplicate observations.

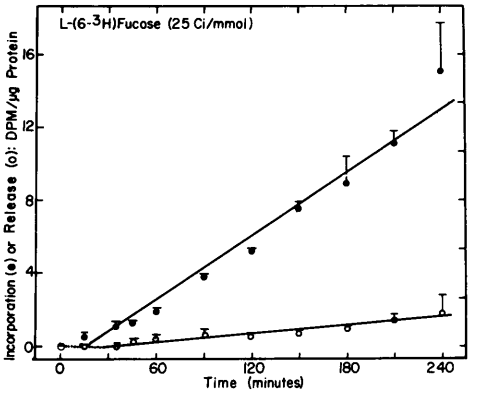

FIG. 7. The temporal pattern of incorporation of L-[6-^{3}H]fucose into glycoproteins and the release of [^{3}H]glycoproteins by rat anterior pituitaries incubated *in vitro*. Each point represents the mean ± SD of triplicate observations.

biosynthetic process. When succinate was substituted for glucose (Fig. 5), an even larger proportion of the incorporated [^{3}H]-GlcNAc was released. Only a small percentage of the [^{3}H]galactose-containing glycoproteins were secreted at 4 hr (Table I) suggesting incorporation primarily into membrane and intracellular glycoproteins. In contrast, a significant percentage of [^{3}H]-fucose-containing glycoproteins were released at 4 hr (Table I). The value observed in the present study is quite comparable to that observed by Todd and Samli (6). Thus, the processes involved in glycoprotein biosynthesis by rat anterior pituitary tissue appear to be consistent with those reported for other tissues (8) and those obtained with rat and bovine pituitary-derived cell-free systems (3–5).

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