Relationships of Thyrotropin to Exophthalmic-producing Substance

PURIFICATION OF HOMOGENEOUS GLYCOPROTEINS CONTAINING BOTH ACTIVITIES FROM [3H]-LABELED PITUITARY EXTRACTS

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SUMMARY

Preparations of thyrotropin which are purified by standard procedures of column chromatography contain several electrophoretically distinct components which have exophthalmogenic activity and contain galactose. Although preparative disc gel electrophoresis can resolve these heterogeneous preparations into individual glycoproteins, several of the resultant components still have both thyrotropic and exophthalmogenic activity. Two such glycoproteins, each with both activities, are homogeneous in the ultracentrifuge, with a molecular weight of 27,000 ± 3,000. They have a similar, if not identical, amino acid composition. Previous suggestions that the determinants for both thyrotropic and exophthalmogenic activity can reside on the same glycoprotein molecule are reinforced by these data.

Terminal galactose residues of glycoproteins may be enzymatically labeled with tritium. This procedure involves a preliminary exposure of the glycoprotein to galactose oxidase and subsequent reduction with (3H) sodium borohydride. Application of this technique has established the terminal nature of galactose in the multiactive but homogeneous preparations obtained by preparative gel electrophoresis. Both the thyrotropic and the exophthalmogenic activities are preserved in the tritiated glycoproteins. The procedure is applicable to crude pituitary preparations, the tritium serving as a convenient marker of the individual glycoprotein molecule.

Thyroid-stimulating hormone derived from mouse transplatable tumors also contains terminal galactose which can be labeled by tritiation. Distinct differences from bovine thyrotropin can otherwise be shown in its sugar composition.

In 1931, Schockaert demonstrated that injections of pituitary extracts produced exophthalmos in ducks (1). Although extensive subsequent work has attempted to distinguish an exophthalmos-producing substance separable from other pituitary hormones, present evidence favoring the existence of a factor distinct from thyrotropin is not completely satisfactory. Claims for the unique nature of these activities are based on the differential solubilities of TSH and EPS in 8% trichloroacetic acid (2), on the separation of both activities with Cm-cellulose and diethylaminoethyl cellulose columns (3, 4), and on the absence of exophthalmogenic activity in concentrates obtained from transplanted mouse tumors which produce a thyrotropic factor (5). Full acceptance of these assertions has been delayed by failure to exhibit a thyrotropin-free exophthalmogenic factor and by conflicting data.

Recent work (6-9) has not confirmed the separation of TSH and EPS activities in bovine pituitary preparations. In these studies, the specific activities of TSH and EPS showed parallel increases during several chromatographic steps. However, since the most highly purified preparations tested in one laboratory contained at least two components on immunoelectrophoresis (6-8) and since the most highly purified preparations from the other laboratory contained several components on starch gel electrophoresis (9), the determinants for TSH and EPS activities could still have been on different glycoprotein molecules (9).

In the present report, previously purified glycoprotein preparations containing both hormonal activities (6-8) are shown to have a terminal galactose which can be tritiated. Tritiated and nontritiated preparations are purified to a greater degree by preparative gel electrophoresis and unique glycoproteins containing tritiated galactose as well as both activities, TSH and EPS, are isolated. Two of the glycoproteins containing both activities are shown to be homogeneous in the ultracentrifuge, to have a molecular weight of 27,000 ± 3,000, and to have a similar, if not identical, amino acid composition.

Mouse tumor TSH is shown to contain terminal galactose which can be tritiated.

The abbreviations used are: TSH, thyroid-stimulating hormone; EPS, exophthalmos-producing substance; Cm- carboxymethyl-.
Crude bovine pituitary thyrotropin was a commercial preparation (Ambinon Organon, Oss, Holland) with a specific activity of 0.6 I.U. TSH per mg. Thyrotropin extracted from transplanted pituitary tumors of mice was a gift from Dr. R. W. Bates. Crude mouse tumor TSH preparations had a specific activity of 1.4 I.U. TSH per mg, whereas purified preparations were 10 I.U. per mg. Galactose oxidase, a units per mg, was purchased from Worthington. Sephadex G-25 and Sephadex G-100 were products of Pharmacia; CM-cellulose and DEAE-cellulose were products of the Reeve Angel Corporation, Clifton, New Jersey. The [%]-sodium borohydride utilized in these experiments was obtained from Nuclear-Chicago; the specific activity of the lot used was 200 mCi per mmole.

PROCEDURE OF [3H]-Labeled Thyrotropin

A technique has previously been described which will uniquely label terminal $d$-galactose residues of ceruloplasmin (21). It involves oxidation of the terminal galactose by the highly specific enzyme, galactose oxidase, followed by reduction with tritiated borohydride (Reaction 1).

\[ \text{HO} \quad \text{CHO} \quad \text{HOR} \quad \xrightarrow{\text{NaBT}^*} \quad \text{HO} \quad \text{CHO} \quad \text{HOR} \]

The following experiment shows that the above procedure (21) will label galactose in partially purified thyrotropin preparations (8-8) and, thus, confirms the fact that such preparations contain a terminal galactose (14).

Galactose oxidase, 5 units, and 10 mg of partially purified bovine TSH, 25 units per mg (6), were dissolved in 2 ml of 0.05 M sodium phosphate buffer at pH 7.0, which was 0.05 M in sodium chloride. Toluene, 0.02 ml, was added and the mixture was incubated at 37° for 24 hours before the reagents were diluted 5-fold by addition of 0.05 M sodium phosphate at pH 7.8, which was 0.05 M in sodium chloride. [%]-Sodium borohydride, 50 μmole, was added and allowed to react for 30 min at room temperature. The tritiated solution was dialyzed at 4° against 5 liters of deionized water. The dialyzing solution, 5 liters of distilled water, was changed twice each day; after 8 days, no radioactive material was detected in aliquots of the dialyze.

The glycoprotein preparation contained $37 \times 10^6$ cpm. A control preparation which was not treated with galactose oxidase had $9 \times 10^6$ cpm after otherwise identical treatment. Evaluation of the neutral sugars indicated that galactose was present in both the control and labeled preparation; however, only the latter was tritiated (Fig. 1). No other neutral sugars were labeled in either preparation.

Less than 1 mole (0.69) of galactose per 28,000 g of glycoprotein was found in these labeled and unlabeled TSH preparations. This result confirms the results of Kim et al. (14); however, acid hydrolysis as applied to ceruloplasmin has been reported to result in at least some destruction of galactose (14, 22). Since galactose in these TSH preparations is terminal, as in ceruloplasmin, an analogous loss might be expected.
the column chromatographic procedures previously described were subjected to the purification procedure to resolve the relationship of terminal galactose to these two factors. Incubation mixtures contained tritiated and nontritiated crude preparations were subjected to the chromatography of tritiated crude TSH preparations. A column, 3 × 120 cm, is equilibrated and eluted with 0.01 M sodium phosphate at pH 6.2. Flow rate is 20 ml per hour. Fractions of 10 ml are collected and monitored for conductivity (A—Δ), absorbance (●—●), and label (○—○). The third peak containing tritium contains salts and nonprotein-bound label. Nontritiated preparations behave identically (6–8). B, CM-cellulose chromatography of tritiated crude TSH preparations. A column, 2 × 25 cm, is equilibrated with 0.01 M sodium phosphate at pH 6.2. The active, salt-free Sephadex eluate, 150 ml, is applied, and the column washed with equilibrating buffer before elution with 1.0 M sodium chloride. Flow rate is 20 ml per hour; 10-ml fractions are collected. Absorbance (●—●) and label (○—○) are monitored. The first peak has no TSH-EPS activity; the peak eluted with sodium chloride is active in both bioassays. Nontritiated preparations separate in identical fashion (6–8). C, DEAE-cellulose chromatography of tritiated crude TSH preparations. A column, 2 × 20 cm, is equilibrated with 0.005 M sodium glycinate buffer, pH 9.5. The desalted active eluate of the CM-cellulose procedure is applied and the column was washed with equilibrating buffer to remove a nonlabeled protein inactive as TSH or EPS. Elution with 0.2 M sodium glycinate, pH 9.5, removes a labeled protein peak active in both bioassays. Flow rate is 20 ml per hour; fractions are 10 ml in volume. Absorbance (●—●) and label (○—○) are monitored. The active fraction from nontritiated preparations elutes in the same position and under identical conditions (6–8).

It is also most important to note that the preparation of TSH which was utilized above was exophthalmogenic and was immunologically heterogeneous (6–8). It contained four major components and one minor component (Rf 0.4, 0.35, 0.25, 0.13, and 0.68, respectively) by analytical disc gel electrophoresis at pH 9.5. The preparations evaluated by Kim et al. were similarly heterogeneous (14, 23–25). The existence of a fractional residue of galactose per 28,000 g of glycoprotein had to be interpreted within these noted limitations (14). The relationship to TSH or to EPS was similarly unclear as was the relationship of terminal galactose to these two factors.

\[ \text{FIG. 1. Identification of tritiated galactose.} \]

The neutral sugar fraction of the tritiated TSH-EPS hydrolysate (A) was cochromatographed with standards. The solvent system is: pyridine-ethyl acetate-water (1.0:3:6:1.15, v/v). Chromatography was for 15 hours with use of a descending technique and Whatman No. 1 filter paper. Movement was from the origin (arrow) to the left. After drying, the radioactivity tracing was recorded with a Vanguard automatic chromatogram scanner. Sugar standards, D-galactose and D-mannose, were subsequently located with silver nitrate reagent (15). The control reaction (B) had no radioactivity coincident with the D-galactose standard.

\[ \text{FIG. 2. A, Sephadex G-25 chromatography of tritiated crude TSH preparations. A column, 3 × 120 cm, is equilibrated and eluted with 0.01 M sodium phosphate at pH 6.2. Flow rate is 20 ml per hour. Fractions of 10 ml are collected and monitored for conductivity (A—Δ), absorbance (●—●), and label (○—○). The third peak containing tritium contains salts and nonprotein-bound label. Nontritiated preparations behave identically (6–8). B, CM-cellulose chromatography of tritiated crude TSH preparations. A column, 2 × 25 cm, is equilibrated with 0.01 M sodium phosphate at pH 6.2. The active, salt-free Sephadex eluate, 150 ml, is applied, and the column washed with equilibrating buffer before elution with 1.0 M sodium chloride. Flow rate is 20 ml per hour; 10-ml fractions are collected. Absorbance (●—●) and label (○—○) are monitored. The first peak has no TSH-EPS activity; the peak eluted with sodium chloride is active in both bioassays. Nontritiated preparations separate in identical fashion (6–8). C, DEAE-cellulose chromatography of tritiated crude TSH preparations. A column, 2 × 20 cm, is equilibrated with 0.005 M sodium glycinate buffer, pH 9.5. The desalted active eluate of the CM-cellulose procedure is applied and the column was washed with equilibrating buffer to remove a nonlabeled protein inactive as TSH or EPS. Elution with 0.2 M sodium glycinate, pH 9.5, removes a labeled protein peak active in both bioassays. Flow rate is 20 ml per hour; fractions are 10 ml in volume. Absorbance (●—●) and label (○—○) are monitored. The active fraction from nontritiated preparations elutes in the same position and under identical conditions (6–8).} \]

Purification of Crude TSH Preparations Containing Tritiated Galactose

Preparative disc gel electrophoresis was used to obtain individual glycoprotein components and thereby resolve the relationship between the hormonal activities. Tritiated and untritiated samples were subjected to the purification procedure to resolve the relationship of terminal galactose to TSH and EPS.

Crude preparations of bovine TSH (Ambinon) were tritiated prior to purification by preparative gel electrophoresis, and was exophthalmogenic and was immunologically heterogeneous (6–8). A typical purification of a labeled tritiated extract is described below in order to show that the behavior of tritiated TSH-EPS glycoproteins is identical with the behavior of nontritiated TSH-EPS glycoproteins (6–8) during these procedures.

**Step 1: Sephadex G-25 Chromatography**—The labeled concentrate was applied to a column, 3 × 120 cm, previously equilibrated with 0.01 M sodium phosphate at pH 6.2. Elution with the same buffer at 20 ml per hour showed the major peaks of protein and radioactivity ahead of the salt front (Fig. 2A). Residual reagent and reagent decomposition products were contained within the salt peak.

**Step 2: CM-cellulose Chromatography**—Fractions 15 to 30 of Step 1 were applied to a column, 2 × 25 cm, equilibrated with 0.01 M sodium phosphate buffer at pH 6.2. The peak eluted with 1.0 M sodium chloride (Fig. 2B) contained radioactivity and was active in the TSH and EPS bioassays. This peak was

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The samples evaluated are from a tritiated preparation. Gel 5, nonabsorbed fraction from Cm-cellulose; Gel 6, purified TSH-EPS, 25 pg, stained with Coomassie blue; and Gel 7, purified TSH-EPS, 25 pg stained with Amido black. The samples evaluated are from a tritiated preparation. A nontritiated preparation is identical at each step. Gels 5 through 7 were loaded with 50 pg of their respective preparations. Gels run with reversed polarity had no other protein staining components. The \( R_f \) 0.68 species, which is predominantly removed in the Cm-cellulose step (Gel 3), is barely visible and is apparently less than 5% of the components in Gel 6.

pooled and desalted by passage through a Sephadex G-25 column, 5 x 50 cm, equilibrated with 0.005 M sodium glycylate at pH 9.5.

The peak eluted before the introduction of 1.0 M sodium chloride contained radioactivity but was inactive in both bioassays and was discarded.

**Step 3: DEAE-cellulose Chromatography**—The desalted active fraction was adsorbed on a DEAE-cellulose column, 2 x 20 cm, previously equilibrated with the 0.005 M sodium glycylate buffer at pH 9.5. The elution rate was 25 ml per hour (Fig. 2C). The fraction eluted with 0.2 M glycyl buffer contained both TSH and EPS, a 10-fold increase in the specific activities of both resulting. Evaluation of the active 0.2 M glycylate eluate by means of acid hydrolysis and paper chromatography indicated that at least 91% of the tritium label was in galactose. Negligible amounts of radioactive material were found in nonadsorbed protein fractions and over 80% of the applied radioactivity was recovered within the active fractions.

Contaminant proteins which eluted before the 0.2 M glycyl application clearly contained negligible radioactivity and were hormonally inactive. The TSH-EPS active peak from a control preparation, which had been subjected to tritiated borohydride reduction without prior galactose-oxidase treatment, was free of radioactivity. For these reasons and since negligible radioactivity was detected elsewhere in the eluate of a control, tritium appears to be a reasonable marker for the TSH and EPS active fractions at this third step.

Analytical disc gel electrophoresis of the chromatographic fractions showed identical protein separations in tritiated and nontritiated preparations (Fig. 3). In both cases the DEAE-cellulose fraction (Fig. 3, Gels 6 and 7) contained four distinct protein-staining species with \( R_f \) values of 0.40, 0.35, 0.25, and 0.13, and one barely visible species with \( R_f \) 0.68.

The active fractions were pooled, dialyzed for 18 hours in 0.001 M ammonium bicarbonate at 2°, and lyophilized. Before lyophilization the TSH specific activity of pooled tritiated preparations was 20 to 25 units per mg. Activity losses incident to lyophilization (24, 26, 27) were similar in tritiated and nontritiated preparations and no significant change on analysis by analytical disc gels was noted as a result of lyophilization. Aliquots of the lyophilized preparations, solubilized in 0.05 M triethanolamine at pH 6.8, were used for preparative gel electrophoresis, where the individual components were resolved.

**Step 4: Preparative Disc Gel Electrophoresis**—Procedures performed in a preparative apparatus, obtained from the Canal Industrial Corporation, Rockville, Maryland. The same pH 9.5 gel system utilized in the above analytical procedures was used for preparative runs. The separating gel, 7.5% in acrylamide, was formed to a height of 4.5 cm; the stacking gel was twice the volume of sample. Samples were applied in the pH 9.5 Tris-glycyl buffer containing 10% sucrose (w/v); they contained 0.1 ml of 0.005% tracking dye, bromphenol blue. A pH 9.5 Tris-glycyl solution (19) served as electrode chamber buffer and elution buffer. With current maintained at 16 ma, total running time was 24 hours. Fractions were collected at a rate of 0.5 ml per min; the 280 nm absorbance was measured in a Beckman DU spectrophotometer. All manipulations were performed at 4°. Individual fractions were analyzed for protein, assayed for TSH activity, and submitted to analytical gel electrophoresis. The results of a typical procedure with the use of 7.5 mg of a tritiated preparation are presented in Fig. 4. Nontritiated preparations behaved in an identical fashion.

Several distinct absorbance areas (II to VI, Fig. 4) were consistently noted to correlate well with tritium-labeled glycoprotein peaks. Although TSH activity could be found in individual fractions at the start (Fraction 38), in the middle (Fractions 73, 80, 87, and 100), and at the end (Fraction 150) of the separation, disc gels of individual fractions indicated the success of the procedure in terms of its separating capacity. Direct analyses of each fraction for exophthalmogenic factor were attempted despite the low sensitivity of the assay. Fractions 45, 80, 87, and 150 had EPS activity.

**Properties of TSH-EPS Glycoproteins Isolated by Preparative Gel Electrophoresis**—The data indicated that distinct glycoprotein species could be isolated by this procedure. The data further suggested that several of the species contained not one but both biological activities, TSH and EPS. In order to confirm these results and to show that each doubly active species was a unique glycoprotein, the individual fractions were grouped, pooled, dialyzed for 18 hours at 2° in 0.001 M ammonium bicarbonate, and concentrated. Concentration was by lyophilization or by ultrafiltration in an Amicon apparatus equipped with a UM-2 membrane. Each fraction was adjusted with 0.1 M Tris-chloride, pH 7.4, assayed for protein, subjected to disc electrophoresis, and tested for TSH and EPS activities. The results are summarized in Table I and Fig. 5. Sample fractions correspond to those outlined on the bottom of Fig. 4. Relatively clean separation of the several glycoprotein species is clearly noted.

\(^2\) Amicon Corporation, Lexington, Massachusetts. The UM-2 membrane retains material exceeding 1000 in molecular weight.
FIG. 4. Preparative disc gel electrophoresis of the galactose tritiated TSH-EPS preparation obtained after Step 3. The eluate is monitored by analyses of absorbance (O--O), and label (O----O) in each fraction. The elution rate is 0.5 ml per min; the fraction volume is 5 to 6 ml. The cross-hatched area denotes fractions containing the tracking dye, bromphenol blue. The analytical disc gel of the starting material is compared with analytical gels of aliquots of the individual fractions obtained. Initial assays are performed on 5-ml aliquots of selected fractions from these peaks. After being lyophilized, these were reconstituted to one-fifth their original volume with quartz-distilled water and were dialyzed at 2° for 6 hours against two portions, 1 liter each, of 0.01 M Tris-chloride buffer at pH 7.4. Each fraction had at this time a pH and molarity, 7.4 and 0.12 M, respectively, which were adequate for the assay in vivo. Fractions

Tritiated preparations from Peak IV–V, assayed directly and within 12 hours of elution from the gel column, had a specific activity of approximately 30 to 35 TSH units per mg of protein. After dialysis and lyophilization, the TSH activities declined 5- to 10-fold to between 3.7 and 5.0 units per mg (Fig. 4); similar decreases were noted in nontritiated preparations. This decrease in activity from initial assays to assays of the concentrates of individual fractions probably reflects a loss related to lyophilization and storage (24, 26, 27). The further decreases noted in TSH assays of the concentrates of pooled fractions (Table I) are at present unexplained; however, the addition of recrystallized bovine serum albumin (0.1% w/v) to the concentrates abolished this decrease and resulted in TSH values analogous to those in Fig. 4.

Of significant interest is the correlation of TSH and exophthalmogenic activities in each of the four single banded species isolated, $R_f$ 0.4, 0.35, 0.25, and 0.13. (The $R_f$ 0.68 species appeared inactive in both assays.) The glycoprotein from Peak IV, $R_f$ 0.25, had the highest TSH specific activity and the highest titer of exophthalmic factor. The second most potent species was the glycoprotein with $R_f$ 0.4; the least potent species were the faster migrating glycoproteins, $R_f$ 0.4 and 0.35.

Details of these experiments are noted in the text. The break in the peaks (Fractions 115 to 144) is an artifact of this particular experiment. A power failure allowed diffusion alone to be the driving force during this period.
TABLE I

| Fractions | Gel no. in Fig. 4 | TSH activity (mU/mg protein) | EPS activity (mU/mg protein) |
|-----------|-------------------|-----------------------------|-----------------------------|
| II B      | 3                 | 80                          | 89                          |
| III A     | 4                 | 49                          | 49                          |
| III B     | 5                 | 103                         | 65                          |
| III C     | 6                 | 37                          | 37                          |
| IV A      | 7                 | 196                         | 196                         |
| IV B      | 8                 | 770                         | 320                         |
| IV C      | 9                 | 834                         | 420                         |
| V A       | 10                | 706                         | 360                         |
| V B       | 11                | 590                         | 330                         |
| V C       | 12                | 15                          | 91                          |
| VI A      | 13                | 52                          | 85                          |
| VI B      | 14                | 190                         | 137                         |
| VI C, D   | 15                | 168                         | 116                         |
| VI E      | 16                | 6                           | 6                           |

Proteins are determined quantitatively by the Lowry reaction with the use of an albumin standard (12). Fractions (Fig. 4) are concentrated as described in the text.

Tritium was not detected elsewhere on the chromatograms. From these experiments it was clear that each of the individual glycoprotein species contained tritium, that the tritium was in galactose, and that the radioactivity accurately and uniquely demarcated the individual glycoproteins (Fig. 6).

The possibility that these species of protein were not homogeneous by criteria other than disc gel electrophoresis or that they represented either polymers or degradation products of a single protein was considered. Disc gel electrophoresis in higher concentrations of acrylamide, up to 20% in the separating gel, did not indicate separations of single banded species into multiple bands. Evaluation of gels using different pH systems also did not yield evidence of additional proteins in the single banded species of Fig. 5; each species ran true when again subjected to electrophoresis after elution from such analytical gels and no new protein bands appeared.

Sedimentation equilibrium studies were performed on the two
glycoprotein species most active in the TSH and EPS assays, i.e. on the glycoproteins from Fractions IV C and VI B, R₀₂₅ and 0.13, respectively (Fig. 7). No evidence of polydispersity was found; the calculated molecular weights were 27,000 ± 3,000 for each species.

The amino acid composition of the different proteins was evaluated (Table II). Although no major differences are noted among fractions of Peaks II, IV, V, and VI, the absence of differences is not unequivocally established since a 5% change could be missed. The high glycine and alanine values may well represent residual contamination from the glycine buffers used in the gel electrophoresis systems.

The amino acid data presented are similar in nature to data presented by other laboratories evaluating TSH preparations (24). Data in all cases are based on similar amounts of protein hydrolyzed, similar nitrogen presumptions, and similar recoveries of standards (approximately 85%). Data obtained in this laboratory with the use of one-tenth the amount of protein, 100 to 250 µg, gave significantly different results yet had better recoveries of the norleucine included as standard. The reasons for these discrepancies may relate to humin formation. These differences will hopefully be resolved during future peptide examinations.

Although the individual fractions were stable during the course of these experiments, as evaluated by disc gel analyses, fractions incubated for prolonged periods in pH 9.5 buffers or fractions repeatedly frozen and thawed showed modifications in electrophoretic behavior. The single banded protein preparations of Fig. 5, after 6 weeks of freezing and thawing in a pH 9.5 buffer, yielded one or several of the other active glycoproteins, i.e. Fraction VI E which contained one glycoprotein with R₀₂₅ and 0.13 now contained glycoproteins with R₀₂₅ and 0.13. Such changes were not seen at 1 to 5 weeks of storage at -10° and

![Graph](http://www.jbc.org/)

**Fig. 7.** Meniscus depletion sedimentation equilibrium experiments with Fractions IV C and VI B, fractions containing the glycoproteins with R₀₂₅ values of 0.25 and 0.13, respectively. Solvent is 0.1 M Tris-Cl, pH 7.4. Protein concentrations were 0.05 (●) and 0.1 (○) mg per ml. Column heights were 3 mm. Centrifugation time was 48 hours; rotor speed was 28,000 rpm. Semilog plots reflect the equilibrium distribution at 25°.

| Component       | III A | III C | IV C | V A  | VI B | VI D | Bates and Condie | Carstens and Pierce |
|-----------------|-------|-------|------|------|------|------|------------------|---------------------|
| Lysine          | 12.9  | 12.1  | 13.1 | 12.8 | 12.7 | 11.8 | 13               | 15-16               |
| Histidine       | 4.4   | 4.3   | 4.1  | 4.2  | 4.5  | 4.3  | 4-5              | 5                   |
| Arginine        | 7.5   | 7.1   | 7.3  | 7.9  | 7.6  | 7.6  | 7-8              | 7                   |
| Aspartic acid   | 13.9  | 13.9  | 14.1 | 13.7 | 14.6 | 13.6 | 12               | 12-14               |
| Threonine       | 13.6  | 12.6  | 14.4 | 13.1 | 13.9 | 12.8 | 14-15            | 15-16               |
| Serine          | 10.5  | 10.5  | 11.1 | 10.9 | 10.0 | 9.8  | 9-10             | 8-10                |
| Glutamic acid   | 15.9  | 15.9  | 16.4 | 14.8 | 15.3 | 14.8 | 13-14            | 14-15               |
| Proline         | 13.9  | 14.0  | 14.7 | 14.3 | 13.9 | 14.4 | 15               | 14-15               |
| Glycine         | 17.9  | 17.6  | 18.9 | 17.8 | 18.5 | 18.7 | 13               | 12-15               |
| Alanine         | 15.2  | 15.8  | 15.6 | 15.7 | 16.0 | 14.7 | 11               | 12                  |
| Valine          | 9.6   | 9.4   | 9.3  | 10.5 | 9.7  | 9.7  | 10-11            | 9-11                |
| Half-cystine    | 14.2  | 15.0  | 13.5 | 14.7 | 14.1 | 12.6 | 15               | 10-15               |
| Methionine      | 4.7   | 4.6   | 5.3  | 4.9  | 5.4  | 4.9  | 5-6              | 5-6                 |
| Isoleucine      | 6.4   | 6.0   | 6.5  | 6.0  | 6.8  | 6.7  | 6               | 6-7                 |
| Leucine         | 8.8   | 8.0   | 8.3  | 8.4  | 7.5  | 8.1  | 9                | 6-8                 |
| Tyrosine        | 8.3   | 8.5   | 9.1  | 8.6  | 8.8  | 8.1  | 8               | 10-11               |
| Phenylalanine   | 6.3   | 6.9   | 6.8  | 6.6  | 6.9  | 7.2  | 7               | 6-7                 |
are present in bovine pituitary TSH (14), quantities per g and relative proportions are different.

Tumor TSH could be labeled with [H]-sodium borohydride after galactose oxidase treatment. The procedure was analogous to that described above. Exact conditions utilized were tumor TSH, 4 mg; initial solubilizing buffer, 4 ml; galactose oxidase, 1 mg; diluting buffer, 20 ml; [H]-sodium borohydride, 5 mCi. Incubation times and conditions were identical as was the water dialysis procedure. A control without added galactose oxidase was used. As with bovine pituitary TSH, tritiation was over 6-fold higher in the galactose oxidase-treated preparation, and analysis of the neutral sugars indicated tritium incorporation in galactose only.

Characterization of the tumor preparation utilized in these experiments has been described in greater detail by Bates and Condliffe (29). Analytical disc gel electrophoresis of the purified tumor preparations did not indicate the presence of more than one protein species, but, compared to bovine pituitary TSH, it showed differences in electrophoretic mobility and diffusion properties. Unlike the bovine TSH preparation, the tumor preparation did not deplete the meniscus when centrifuged at 28,000 rpm in the Spinco model E ultracentrifuge.

**DISCUSSION**

This work shows unequivocally that galactose residues are unsubstituted in their carbon 6 positions. The terminal nature of galactose is therefore indicated in both the bovine pituitary and mouse tumor preparations (21). The possibility of galactose destruction during hydrolyses (14, 22) and the heterogeneous nature of the hydrolyzed preparation made it impossible to determine quantitatively the molar ratio of terminal galactose to TSH-EPS glycoprotein in partially purified TSH-EPS preparations. In the single banded preparations subsequently obtained, each component glycoprotein was shown to have terminal galactose (Figs. 5 and 6). Although quantitative determination was not possible on the homogeneous preparations, future studies with larger amounts of glycoprotein should resolve this point. These results do not preclude the existence of additional galactose having nonterminal linkages in one or all of the isolated glycoproteins.

Disc gel electrophoresis studies (Fig. 3, Gels 6 and 7) of tritiated and nontritiated preparations have clearly confirmed the heterogeneous or multicomponent nature of purified material after several steps of chromatographic purification (6-8, 23). Several of these components, two of which are homogeneous by criteria of equilibrium ultracentrifugation as well as disc gel electrophoresis at several pH values and acrylamide concentrations, have been isolated by preparative disc gel electrophoresis. Since each homogeneous preparation contains both TSH and exophthalmogenic activity, it is evident that a single glycoprotein molecule can be responsible for both activities. Although the amino acid analyses of the several active fractions show few quantitative differences, alterations in peptide composition or in amide content have not been excluded. Evaluation of the carbohydrate content is of obvious interest, but the amount of material available did not allow complete analysis.

The present work supplements previous work by Carsten and Pierce (23) and by Dedman et al. (9). The experiments confirm that analogous TSH preparations are exophthalmogenic yet indicate that the several individual components can be isolated preparatively by preparative gel electrophoresis, that each component has both TSH and EPS activity, and that each of these components contains terminal galactose.

Modification of the carbohydrate content or polypeptide structure of a factor uniquely active as TSH could have yielded one or several of the glycoproteins having both TSH and EPS activity. Although no unique TSH factor has been shown in these experiments and although these glycoproteins may well be preparative artifacts (28), such a hypothesis is attractive if one assumes a pituitary origin for exophthalmos in Grave's disease and accounts for the very low levels of TSH found in the sera of these patients (30, 31). The existence of such doubly active glycoproteins does not, however, eliminate the idea that pituitary hormones may have no role at all in the pathogenesis of exophthalmos in man. A long acting thyroid-stimulating hormone has been found which is not synthesized by the pituitary and has the chemical criteria of immunoglobulins (32, 33); a nonpituitary factor might also be responsible for human exophthalmos.

One major difficulty in evaluating the determinants of TSH or EPS activity, as well as their relationships, has been the reliable purification of a single component, homogeneous, preparation (23). Differences and differences in the results of purification among the several groups of workers have been attributed to the structural instability of the glycoprotein (23) and to the poor reliability of bioassays, particularly that for EPS (9). The present report suggests that utilization of the galactose-labeling procedure may aid future purifications. Although other glycoproteins such as luteinizing hormone appear to be tritiated in crude preparations (Fig. 2B), TSH EPS molecules remain as the only significantly labeled glycoproteins after the first few purification steps (Figs. 2C, 4, and 6). The determination of radioactivity can supplement the time-consuming bioassays of subsequent steps.

Although injections of mouse pituitary tumor TSH (10 i.u. per animal) failed to produce exophthalmos in fishes, its lack of exophthalmogenic activity cannot be used to exclude the possibility that normal pituitary factors exhibit both activities. The biological specificity presently reported confirms earlier experiments using this same material (5); however, it is already known that tumor TSH differs from the bovine preparation in hexose composition, no galactosamine being detected (25). The present experiments indicate a significant difference in hexose composition despite the existence of terminal galactose. The different hexose composition previously reported for tumor TSH (29) does not negate this point but intensifies it. The difference in galactose content, 6 moles (29), as opposed to 1 mole (present report), per 28,000 g of tumor TSH, may relate to differences in the preparation which was assayed or to differences in the assay procedure which was used, i.e., gas-liquid chromatography or paper chromatography. Previously reported physicochemical differences (26) may well reflect additional differences in the protein structure (29). In sum, when the relationships of TSH and EPS are considered, an extremely tenacious link exists between the bovine and mouse tumor factors, one which cannot at all argue for the unique nature of EPS (5).
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