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Characterization of Phosphate Residues on Thyroglobulin*

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Follicular 19 S thyroglobulin (molecular weight 660,000) from rat, human, and bovine thyroid tissues contains ~10–12 mol of phosphate/mol of protein. These phosphate residues can be radiolabeled when rat thyroid hemilobes, FRTL-5 rat thyroid cells, or bovine thyroid slices are incubated *in vitro* with [^{32}P]phosphate. Thus labeled, the [^{32}P]phosphate residues co-migrate with unlabeled 19 S follicular thyroglobulin on sucrose gradients and gel filtration columns; are specifically immunoprecipitated by an antibody preparation to rat or bovine thyroglobulin as appropriate; and co-migrate with authentic 19 S thyroglobulin when subjected to analytic or preparative gel electrophoresis. Tunicamycin prevents ~50% of the phosphate from being incorporated into FRTL-5 cell thyroglobulin. Approximately one-half of the phosphate in FRTL-5 cell or bovine thyroglobulin can also be released by enzymatic deglycosylation and can be located in Pronase-digested peptides which contain mannose, are endo-β-*N*-acetylglucosaminidase H but not neuraminidase-sensitive, and release a dually labeled oligosaccharide containing mannose and phosphate after endo-β-*N*-acetylglucosaminidase H digestion. The remainder of the phosphate is in alkali-sensitive phosphoserine residues (3–4/mol of protein) and phosphotyrosine residues (~2/mol of protein). This is evidenced by electrophoresis of acid hydrolysates of ^{32}P-labeled thyroglobulin and by reactivity with antibodies directed against phosphotyrosine residues. The phosphoserine and phosphotyrosine residues do not appear to be randomly located through the thyroglobulin molecule since approximately 75–85% of the phosphotyrosine and phosphoserine residues were recovered in a ~15-kDa tryptic peptide or a ~24-kDa cyanogen bromide peptide, each almost devoid of carbohydrate. ^{31}P nuclear magnetic resonance studies of bovine thyroglobulin confirm the presence and heterogeneity of the phosphate residues on thyroglobulin preparations.

The thyroid is composed of large numbers of follicular structures, each of which is formed by an array of cells surrounding a central cavity or lumen (1–5). Thyroglobulin is the major biosynthetic product of the thyroid cells and is stored in the luminal cavities of the follicles after its synthesis on polysomes (1–5). It is the macromolecular precursor of the thyroid hormones, triiodothyronine and thyroxine.

Glycosylation, a post-translational modification of thyroglobulin which occurs in addition to iodination (7–12), plays an important role in membrane recognition phenomena related to the follicular storage process as well as in the structure of the protein (13–23). In the course of studies aimed at further defining the role or structure of the carbohydrate moieties of thyroglobulin, another post-translational modification of the thyroglobulin molecule was uncovered, phosphorylation (21, 24–27).[1,2] The nature of the phosphate residues has been unclear. Thus, some reports suggested they could be carbohydrate-linked (21, 24, 26).[1] Others indicated the presence of alkali-sensitive phosphoserine residues (25, 27)[2]; and still others (21)[2] indicated phosphotyrosine residues were present.

In the present report we show that bovine, human, and rat thyroglobulin preparations have ~10–12 mol of phosphate/mol of thyroglobulin (660 kDa) and that the phosphate is divided between the B carbohydrate moiety (~50%), phosphoserine residues (~30%), and phosphotyrosine residues (~20%). The report also shows that thyroglobulin preparations have an activity which can hydrolyze phosphate residues, particularly their own carbohydrate-linked residues. This activity may explain why this phosphorylation was not recognized as a post-translational modification of thyroglobulin until recently (21, 24–27)[1,2] and why some of these reports have not detected the amounts or types of phosphate described herein.

MATERIALS AND METHODS

Cell Cultures—FRTL-5 cells (ATCC CRL 8305) are a continuous, cloned line of functioning cells (28) which are maintained in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and a mixture of six hormones or growth factors, *i.e.* insulin, thyrotropin, glycyl-L-histidyl-L-lysine, human transferrin, cortisone, and somato-

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[1] This work was presented at the Second European Congress on Cell Biology (Neumuller, W., and Herzog, V. (1986) *Acta Biol. Hung.* **37,** Abstr. 334, suppl. 137).

[2] This work was presented at the 15th Annual Meeting of the European Thyroid Association (Acquaviva, A. M., Tassi, V., Liquoro, D., Gallo, A., Kohn, L. D., and Consiglio, E. (1986) *Ann. Endocrinol.* **47,** 38

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statin (28, 29). Cultures used for thyroglobulin production were in their late log phase of growth.

Thyroglobulin Preparations—Follicular 19 S bovine, human, or rat thyroglobulin was isolated by salt extraction of sliced, fresh thyroid glands. The thyroglobulin in these extracts or in the media of FRTL-5 rat thyroid cell cultures was purified by ammonium sulfate precipitation (1.4–1.8 M) and gel filtration of the solubilized precipitate over Sepharose CL-6B or Sephacryl S-300 (Pharmacia P-L Biochemicals) (16–20). All procedures were carried out at 0–4 °C; phosphodiesterase and/or phosphatase inhibitors, 0.5 M 3-isobutyl-1-methylxanthine (IMX),[3] and/or 150 mM phenyl phosphate, respectively, were added to buffers where indicated. The gel filtration procedure used 0.1 M Tris-chloride, pH 7.6, rather than the phosphate buffer usually present (16–20). Immediately prior to each experiment, the 19 S thyroglobulin preparation was rechromatographed on Sepharose CL-6B in 0.1 M Tris-chloride, pH 7.6, to insure homogeneity; the 19 S value was confirmed by both Model E (Beckman) and sucrose density gradient centrifugation procedures (see below).

Where noted the thyroglobulin was further purified by immunoprecipitation with anti-rat thyroglobulin. Alternatively, or in addition, it was purified by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and reducing reagent (β-mercaptoethanol) followed by overnight electroelution of the 330-kDa band into dialysis bags.

FRTL-5 rat thyroid cells, rat thyroid hemilobes, or bovine thyroid slices were used to prepare *in vitro* radiolabeled thyroglobulin. To radiolabel thyroglobulin in FRTL-5 cells, approximately 0.5×10^6 cells were "starved" by incubating them for 12 h in Coon's modified F12 medium containing 5% calf serum, a six-hormone mixture including insulin and thyrotropin but no methionine and no phosphate. Pilot experiments indicated that the bicarbonate in the media was sufficient to maintain the pH of the media at 7.4 in the presence of a 5% CO_2 gassing mixture; the addition of 20 mM Hepes, pH 7.4, did not alter the results. Carrier-free [^{32}P]phosphate (40 mCi/ml, Amersham Corp.) and/or [^{35}S]methionine (970 Ci/mM, Amersham Corp.), 1.0–1.4 mCi and 150 μCi, respectively, were added in 3 ml of the same medium with the exception that the calf serum was reduced to 0.5%. Cells were then incubated 24 h at 37 °C before media and cells were separately harvested. Separate experiments showed that ^{32}P-radiolabeling of thyroglobulin in the media was measurable within 30 min, increased linearly over 3–4 hr, and was not associated with an apparent loss of cell viability (trypan blue exclusion), thyroglobulin production, or iodide uptake over a 24-h period.

In other experiments, 250–500 μCi of D-[2-^{3}H]mannose (10–20 Ci/mM, Amersham Corp.) was added with the [^{32}P]phosphate. In such experiments, a similar radiolabeling procedure was used, with the exceptions that (*a*) the starvation period was 4–5 h and (*b*) medium with either no or one-tenth the usual concentrations of glucose and phosphate was used. The incubation period for radiolabeling in these experiments lasted 8–16 h and was followed by an additional 5 h to overnight incubation wherein the medium was supplemented with unlabeled glucose and phosphate in sufficient concentrations to make complete medium. This modified starvation protocol was used since cells could not survive 36 h of glucose starvation.

Where noted, tunicamycin, 1 μg/ml, was added to the cell incubations during the starvation period and during the subsequent incubation period.

To radiolabel thyroglobulin in rat thyroid hemilobes or fresh bovine thyroid slices, 10–20 hemilobes or an equivalent mass of sliced bovine thyroid tissue was incubated in 6 ml of either phosphate-free or phosphate- and methionine-free Coon's modified F12 medium for 40 min. The medium was removed, replaced with 6 ml of the same medium containing 1.5 mCi [^{32}P]phosphate without or with, respectively, 150 μCi [^{35}S]methionine, and the incubation continued for 90 min at 37 °C in a 95% O_2, 5% CO_2 environment. The rat hemilobes or bovine slices were homogenized and thyroglobulin isolated by an ammonium sulfate precipitation procedure (15–20) followed by gel filtration chromatography, immunoprecipitation, and gel electrophoresis as noted in individual experiments.

Deglycosylated ^{32}P-labeled FRTL-5 thyroglobulin was prepared by adapting previously described procedures (16–20). Thus, a lyophilized, radiolabeled thyroglobulin preparation (0.5–3 mg of protein) was treated with 1.0 unit of *Vibrio cholera* neuraminidase (Boehringer Mannheim) for 6 h at 37 °C in 1 ml of 0.1 M sodium acetate, pH 5.6, containing 1% crystalline (five times) bovine serum albumin. The incubation was continued at 37 °C for 3 h after the addition of 2.0 units/ml of *Aspergillus niger* β-galactosidase (Sigma) and for an additional 4 h after the addition of 5.0 units/ml of Jack Bean α-mannosidase (Sigma). Toluene, 2–3 drops, was present throughout the incubation. The deglycosylated, ^{32}P-labeled thyroglobulin was recovered by precipitation with 20% trichloroacetic acid and the residual radioactivity measured. Control experiments were performed with boiled enzyme preparations or with no enzyme present.

Alkali treatment of the ^{32}P-labeled thyroglobulin was performed in 1.0 N NaOH at room temperature for 60 min. After alkali treatment, the thyroglobulin was again collected for measurement of residual radioactivity by precipitation with 20% trichloroacetic acid.

Immunoprecipitation and Gel Electrophoresis Analyses of Thyroglobulin Preparations—Antisera to rat, bovine, or human thyroglobulin were produced in rabbits as previously described (30, 31). Immunoprecipitation of the thyroglobulin used a procedure adapted from Kessler (32), which included a preadsorption step with a preimmune serum and protein A before immunoprecipitation using the appropriate antibody in the presence of protein A. Approximately 300-μl aliquots of column eluates or FRTL-5 cell media diluted ~1:1 in 0.1 M Tris-chloride, pH 7.4, containing 0.15 M sodium chloride, 5 mM EDTA, 1% deoxycholate, 1% Triton X-100, and 50 μg/ml each of phenylmethylsulfonyl fluoride, TPCK, and TLCK were incubated with 30 μl of normal rabbit serum for 10 min at 23 °C before protein A, 40 μl, was added. After 30 min the incubation mixture was centrifuged and the supernatant recovered. Approximately 30 μl of an antisera to thyroglobulin was added to the supernatant and the mixture incubated 30 min at 23 °C before an additional aliquot of protein A, 40 μl, was added. The incubation was continued 0–4 °C overnight; the immunoprecipitated pellet was recovered by centrifugation and was washed five times with incubation buffer without phenylmethylsulfonyl fluoride, TPCK, or TLCK.

Antisera to phosphotyrosine residues were kindly provided by Dr. C. R. Kahn (Joslin Diabetes Center, Boston, MA) and by Dr. P. Comoglio (Department of Histology and Embryology, University of Torino Medical School, Torino, Italy). When using these antisera, 2–4 mg of IgG were added/500 μl volume containing 10 μg of [^{35}S] methionine-labeled FRTL-5 cells or ^{125}I-labeled bovine thyroglobulin.

Gel electrophoretic analyses in 0.1% SDS were carried out using the Laemmli procedure (33) and 4, 6.5, 7.5, 15%, or gradient (7–18%) acrylamide slab gels, as noted. Samples were incubated with β-mercaptoethanol before electrophoresis (33). Radiolabeled molecular weight standards were from Amersham Corp.; unlabeled molecular weight standards were from Pharmacia P-L Biochemicals. The protein was visualized by Coomassie Blue staining; radioactivity was visualized on autoradiographs which were allowed to develop overnight at −80 °C unless otherwise noted. Kodak X-Omat AR films and intensifying screens were used.

Pronase Digestion of Thyroglobulin and Endo-H Treatment of the Resultant Peptide Fractions—Pronase (Behring Diagnostics) digestion of immunoprecipitated, radiolabeled FRTL-5 cell thyroglobulin preparations adapted a previous procedure with respect to conditions and enzyme concentrations (34). Digestion was performed after immunoprecipitated pellets were dissolved in 0.5 ml of 0.1 M Tris-chloride, pH 8.0, containing 10 mM $CaCl_2$. The same procedure was used for 500-mg amounts of the bovine thyroglobulin preparations (100 mg/ml). In each case the Pronase solutions, 10 mg/ml and in 0.1 M Tris-chloride, pH 8.0, containing 0.01 M $CaCl_2$, were preincubated at 37 °C for 2 h to destroy any glycosidase activity. Digestion was performed in the presence of 150 mM phenyl phosphate, a phosphatase substrate added to prevent hydrolysis of radiolabeled phosphate by any potential phosphatase contaminating the Pronase or thyroglobulin preparation. Pronase digestion was at 60 °C, in the presence of toluene, and for 36 h. Aliquots of the Pronase solution were added at 0, 6, 24, and 36 h as appropriate.

Chromatography of the Pronase digest of FRTL-5 cell thyroglobulin used a Bio-Gel P-6 (200–400 mesh) column which was 186 × 1.0 cm. Chromatography of the bovine thyroglobulin digest used a 186 × 2.5-cm column. In both cases the columns were equilibrated in 0.1 M Tris-chloride, pH 8.25, and elution volumes of 0.9 or 9 ml were collected at a flow rate of 10–20 ml/h; 150 mM phenyl phosphate was included in the elution buffer as noted in individual experiments.

[3] The abbreviations used are: IMX, 3-isobutyl-1-methylxanthine; endo-H, endo-β-*N*-acetylglucosaminidase H; ^{31}P NMR, ^{31}P nuclear magnetic resonance; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; HPLC, high pressure liquid chromatography; [Ser(P)], phosphoserine; [Thr(P)], phosphothreonine; [Tyr(P)], phosphotyrosine, IGF-I, insulin-like growth factor I; IgG, immunoglobulin G.

Fractions 50–100, which will be shown to contain ^{32}P- and [^{3}H] mannose-labeled peptides, were pooled, desalted by passage through Sephadex G-25 (Pharmacia P-L Biochemicals) in distilled water, and lyophilized. This material was divided in several aliquots, portions of which served as controls or were treated with endo-β-*N*-acetylglucosaminidase (endo-H) or *V. cholera* neuraminidase.

Endo-H digestion adapted the procedure of Godelaine *et al.* (35), *i.e.* incubation was at 37 °C for 20 h in a final volume of 250 μl containing 0.5 M sodium citrate, pH 5.5, plus 5 millinunits endo-H (30 units/ml). The endo-H was from Miles Laboratories Inc. or was the kind gift of Dr. Frank Maley, New York Public Health Service, Albany, NY. The enzyme was added in 5-μl aliquots, each containing 2.5 milliunits, at 0 and 12 h of the incubation period. *V. cholera* neuraminidase (Behring Diagnostics) digestion was with 1000 units for 15 h at 37 °C in 0.1 M sodium acetate, pH 5.5, containing 10 mM $CaCl_2$ and 0.4% 5 × recrystalized bovine serum albumin. The final volume was 1.5 ml. In both the case of the endo-H and *V. cholera* neuraminidase digestion, the incubation mixtures also contained 1 drop of toluene. At the end of the incubations, the digests were rechromatographed on the Bio-Gel P-6 columns using the procedures and conditions detailed above. Control incubations, containing all components except the noted enzyme, were incubated identically to the experimental samples and were identically chromatographed.

Tryptic and Cyanogen Bromide Phosphopeptides—Tryptic digestion of the radiolabeled FRTL-5 cell thyroglobulin was performed in the presence of purified, unlabeled rat thyroglobulin, 1 mg/ml, as carrier. The digestion used TPCK-trypsin (Worthington) at a concentration of a 10 μg/mg thyroglobulin; digestion was in 20 mM Tris-chloride, pH 8.2, at 37 °C for 180 min. The phosphopeptides were separated using a Gilson high pressure liquid chromatography system equipped with a TSK G3000 SW column. The tryptic phosphopeptide mixture, 100 μl/sample, was applied to the column and eluted at a flow rate of 1.0 ml/min. The mobile phase was composed of 0.1 M ammonium bicarbonate, pH 7.8. Fractions of 100 μl were collected and the first 100 fractions from four such columns were individually pooled. Absorbance at 230 nm was measured before a 40-μl aliquot was used to measure radioactivity. Appropriate samples were subjected to phosphoamino acid analysis.

Cyanogen bromide (CNBr) treatment of phosphorylated thyroglobulin was performed essentially as described by Gross and Witkop (36). Treatment was again in the presence of a carrier: unlabeled rat thyroglobulin, 1 mg/ml. Treatment used 10 mg of CNBr/ml of thyroglobulin and was in 70% formic acid. The mixture was gently shaken overnight, in the dark, at room temperature. After being diluted with H_2O (1/10) and lyophilized, the suspension was reconstituted in 40–50 μl of Laemmli buffer containing β-mercaptoethanol (33). It was neutralized with 0.1 N NaOH, boiled for 2 min, and centrifuged for 5 min at 10,000 rpm in a Beckman Microfuge. A 30-μl aliquot of the supernatant was then subjected to 15% SDS-polyacrylamide gel electrophoresis.

Acid Hydrolysis and Analysis of Phosphoamino Acids—The phosphorylated thyroglobulin preparations were dialyzed against 50 mM ammonium acetate, lyophilized, and hydrolyzed. Acid hydrolysis was performed according to Hunter and Septon (37) in 6 N HCl for 2 h at 110 °C in *in vacuo* sealed tubes. Twice, the hydrolyzed samples were diluted with 50 mM ammonium bicarbonate, pH 7.8, and lyophilized; they were then dissolved in 10 or 20 μl of electrophoresis buffer (pyridine/acetic acid/water (10/100/890 v/v), pH 3.5) containing 0.1 μM each of unlabeled phosphoserine [Ser(P)], phosphothreonine [Thr(P)], and phosphotyrosine [Tyr(P)] standards. Electrophoresis used 0.25-mm cellulose thin layer plates (Machery Nalgene) and was at 1000 V for 60 min at 10 °C (38). Standards were visualized by ninhydrin staining; autoradiographs were allowed to develop at −80 °C on Kodak Y-Omat AR films with intensifying screens.

Immunoblotting—A tryptic digest of thyroglobulin was subjected to SDS-polyacrylamide gel electrophoresis using a 7–18% gradient gel. Western blots were performed at pH 8.5 using a Bio-Rad apparatus and a buffer consisting of 25 mM Tris-chloride, 190 mM glycine, and 20% methanol. The proteins were transferred to 0.45-μm nitrocellulose sheets (Schleicher & Schuell) by applying a current of 30 mA for 12 h at 4 °C. At the end of the procedure, the nitrocellulose sheets were washed twice with phosphate-buffered saline, pH 7.4, and were incubated at 45 °C for 60 min in 20 mM Tris-chloride, pH 7.4, containing 150 mM NaCl, 2 mM EDTA, 2.5% bovine serum albumin, 0.08% Ficol 400, and 0.02% polyvinylpyrrolidone (39). Immunoreactions were performed in the same buffer with 0.05 mg/ml purified IgG from the anti-phosphotyrosine immune and preimmune sera. At the end of the incubation, the nitrocellulose sheets were washed three times with the incubation buffer diluted 1:10. Bound antibody was revealed by incubation with 10^5 cpm of ^{125}I-labeled protein A (Amersham Corp.) for 60 min at room temperature. The excess radioactivity was removed by exhaustive washing in the diluted incubation buffer. The dried blots were exposed to Kodak X-Omat AR films with intensifying screens as above.

Other Procedures—Phosphate assays of unlabeled thyroglobulin preparations used the procedure of Bartlett (40). Samples were prepared for the assay by both Sephacryl S-300 chromatography and extensive dialysis (2 days, at least four changes, 2,000-fold volume excess) in 0.1 M Tris-chloride, pH 7.5. Assays used the dialysis buffer as a control. Labeled and unlabeled preparations had similar phosphate values.

The phosphorus content of radiolabeled preparations of thyroglobulin from FRTL-5 cell or slice experiments was estimated using the specific radioactivity of [^{35}S]methionine and [^{32}P]phosphate after recovery of the thyroglobulin by immunoprecipitation. The specific activity of the ^{32}P used in these experiments was 9900 Ci/mg atoms and that of [^{35}S]methionine was 800 or 1200 Ci/mmol (Amersham Corp.). The values of ^{32}P- and [^{35}S]methionine incorporated into thyroglobulin were corrected for cross-channel spillover and for decay of ^{32}P; dpm calculations were made using a quenched, standard curve program from Beckman. [^{35}S]Methionine incorporated into the sample protein reflects the total protein concentration; the molar concentration of thyroglobulin being estimated on the basis of 85 methionine residues/mol of thyroglobulin (41). The phosphate concentration was measured on the basis of ratio of the dpm in the protein and the mg atoms of ^{32}P determined from the specific activity.

Sucrose density centrifugation used procedures described (42). Ultracentrifugation analyses to identify 19 S thyroglobulin were at 25 °C and were performed after thyroglobulin was dialyzed against 0.1 M potassium phosphate, pH 7.0 (43).

In the preparation of thyroglobulin, protein concentration was measured by absorption at 280 nm ($E^{1\%}_{280}$ = 10.0) (16–20). In all other experiments protein was measured using a colorimetric assay (44), crystalline bovine albumin being the standard.

Assays of neutral sugars were performed using the anthrone procedure (45) or a Beckman auto analyzer adapted for sugar analysis (46). In the latter experiments mannose was discriminated from glucose by the absence of glucose oxidase sensitivity (47).

^{31}P Nuclear Magnetic Resonance (NMR) Studies—19 S follicular bovine thyroglobulin used for ^{31}P NMR studies was prepared as described above, both in the presence and absence of 0.5 M 3-isobutyl-1-methylxanthine. These were used fresh or after storage at −70 °C for 1–6 months. Immediately before use, the thyroglobulin preparation was rechromatographed over a Sepharose CL-6B column equilibrated with Tris-chloride, pH 7.5, and was concentrated to ~100 mg/ml using an Amicon microconcentrating apparatus and an XM-50 filter. About 5 ml of this stock thyroglobulin preparation was further concentrated using the Amicon apparatus and a UM-2 filter to yield a protein-free filtrate and a residual solution of ~200 mg/ml thyroglobulin.

^{32}P NMR spectra were recorded on a Varian XL-300 (121.0 MHz) spectrometer using either a 10- or a 5-mm broad band probe. All spectra were recorded with proton noise decoupling. In general, 50,000–200,000 free induction decay signals were acquired using 8-kDa data points, a 6000 Hz spectral width, a 45 °C pulse angle and 1-s repetition times. A 10–20 exponential line broadening was used prior to Fourier transformation. The buffer for ^{31}P NMR measurements contained 10% D_2O for field/frequency lock. Sample temperature was controlled at 22 ± 1 °C. ^{31}P chemical shifts were reported in parts/million with respect to the inorganic phosphate signal (either internal or external).

RESULTS

Phosphate Residues on Thyroglobulin

Phosphate Residues on Unlabeled Follicular Thyroglobulin Preparations from Bovine, Rat, or Human Thyroid Tissues—When preparations of follicular thyroglobulin were obtained by classic salt extraction procedures from bovine, human, and rat thyroid tissues and were purified by chromatography and rechromatography on Sepharose CL-6B or Sephacryl S-300 in 0.1 M Tris-chloride rather than 0.1 M phosphate (16–20), ~10 mol of phosphate/mol of thyroglobulin could be detected

by chemical analysis (Table I). Exhaustive dialysis in 0.1 M Tris-chloride containing 6 M urea or 6 M guanidine-hydrochloride did not alter these results.

Phosphate Residues in Thyroblogulin Preparations from Rat Thyroid Hemilobes, Rat Thyroid FRTL-5 Cells, or Bovine Thyroid Slices Can Be Identified By Radiolabeling in Vitro—The phosphate residues on thyroglobulin could also be demonstrated in salt-extracted follicular thyroglobulin from rat thyroid hemilobes or bovine thyroid slices which had been incubated with [^{32}P]phosphate and [^{35}S]methionine. In each case, sucrose density gradient centrifugation of the salt-extracted thyroglobulin showed coincident peaks of the two radiolabels migrating at the exact position of unlabeled 19 S thyroglobulin, the sedimentation value of which was established by Model E ultracentrifugation. The ^{32}P radiolabel also cochromatographed with unlabeled 19 S thyroglobulin on Sepharose CL-6B and was completely and specifically precipitated with anti-rat or anti-bovine thyroglobulin as appropriate (Fig. 1). The immunoprecipitated rat or bovine thyroglobulin labeled only with ^{32}P co-migrated with authentic iodinated bovine 19 S thyroglobulin and a 330-kDa marker when subjected to SDS-gel electrophoresis under reducing conditions (Fig. 1, *inset*). Using specific radioactivity values and appropriate calculations, the labeled rat and bovine thyroglobulin preparations, respectively, were found to have ~11.5 and 12.4 ± 1 mol of phosphate/mol of thyroglobulin (660 kDa). These values compare favorably with the data in Table I.

Rat FRTL-5 cells (ATCC CRL 8305) are a continuously growing cloned line of normal Fisher rat thyroid cells which synthesize and secrete thyroglobulin into the media (17, 21, 24, 28, 29). FRTL-5 cells were grown in the presence of [^{32}P] phosphate, [^{35}S]methionine, or [^{3}H]mannose as well as in combinations of these radiolabels (see "Materials and Methods"). As was the case for the ammonium sulfate purified, 19 S follicular thyroglobulin salt extracted from rat hemilobes (see above), ^{32}P-radiolabeled, ammonium sulfate-purified, FRTL-5 cell media thyroglobulin co-migrated with authentic unlabeled 19 S thyroglobulin both on sucrose gradients (not shown) and Sepharose CL-6B gel filtration columns (Fig. 2). The ^{32}P-labeled 19 S thyroglobulin fraction was completely immunoprecipitated by anti-rat thyroglobulin preparations after Sepharose chromatography (Fig. 2); it also co-migrated with authentic unlabeled thyroglobulin on gel electrophoretograms run in the presence of SDS and mercaptoethanol (Fig. 2, *inset*). Using specific radioactivity values and appropriate calculations, the labeled rat FRTL-5 cell thyroglobulin preparations were found to have ~12 ± 0.5 mol of phosphate/mol of thyroglobulin (660 kDa).

TABLE I

Phosphate content of bovine, human, and rat 19 S follicular thyroglobulin preparations

Measured chemically using the procedure of Bartlett (40); values are presented ±S.D. The molecular weight of thyroglobulin used in these calculations was 660,000.

Preparation no. or source	Mol phosphate/mol thyroglobulin
Bovine thyroglobulin	
1	10.0 ± 0.3
2	9.5 ± 0.4
3	10.4 ± 0.4
Human thyroglobulin	
1	8.9 ± 1.0
2	9.8 ± 0.6
Rat thyroglobulin	
1	10.4 ± 0.3
2	9.9 ± 0.4

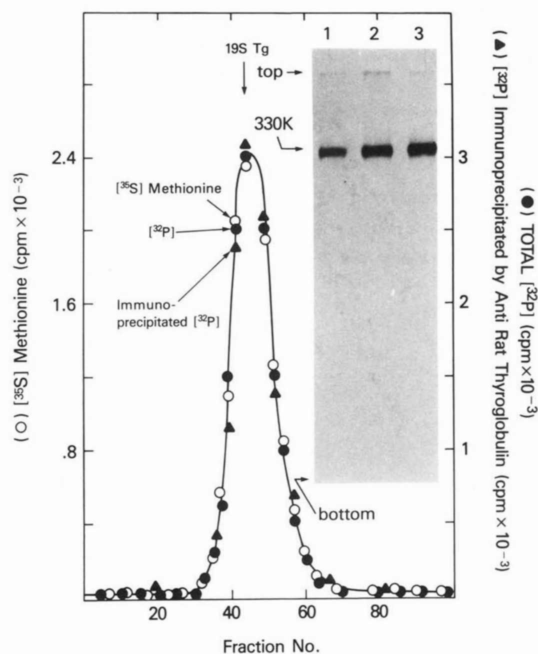

FIG. 1. **Sepharose CL-6B chromatography and immunoprecipitation of the rat thyroglobulin preparation radiolabeled by incubating hemilobes with [^{32}P]phosphate and [^{35}S]methionine.** The thyroglobulin was salt extracted, subjected to sucrose density centrifugation on a 5–28% gradient, and the pooled fractions co-migrating with authentic 19 S thyroglobulin chromatographed on a Sepharose CL-6B column after Sephadex G-25 chromatography in distilled water and lyophilization. The column was equilibrated and eluted in 0.1 M potassium phosphate, pH 7.5. To each fraction was added an antiserum to thyroglobulin and protein A after a precipitation with a control preimmune serum and protein A. After incubation at 37 °C for 2 h, the pellets were precipitated by centrifugation, washed twice with buffer, and the specific immunoprecipitable ^{32}P radioactivity determined. Authentic 19 S thyroglobulin elutes at the position noted. *Inset*, gel electrophoresis in SDS, under reducing conditions, of radioiodinated, authentic 19 S bovine thyroglobulin (*lane 1*) and the ^{32}P-labeled rat (*lane 2*) and bovine (*lane 3*) thyroglobulin formed, respectively, during *in vitro* incubations of rat hemilobes or bovine thyroid slices with [^{32}P]phosphate. The radiolabeled thyroglobulin preparations were immunoprecipitated by antibodies to rat or bovine thyroglobulin as above. Gel electrophoresis used the Laemmli procedure (33) and a 4% gel; the immunoprecipitates were prepared for electrophoresis by dissolving them in the running buffer containing SDS and β-mercaptoethanol (33). The position of a 330-kDa molecular weight marker is noted. The iodinated bovine thyroglobulin was prepared as previously described (16). Autoradiograms were developed for 24 h. *K*, kDa.

Tunicamycin (1 μg/ml), present during the labeling incubation, did not significantly diminish [^{35}S]methionine incorporation into the 330-kDa immunoprecipitated thyroglobulin moiety purified from the media of the FRTL-5 cells (Fig. 3, *lane 3 versus lane 1*; also Table II). Tunicamycin did, however, significantly decrease, but not abolish, the ^{32}P radiolabel incorporation into the 330-kDa immunoprecipitated thyroglobulin moiety purified from the media (Fig. 3, *lane 4 versus lane 2*; also Table II). In experiments using tunicamycin wherein the media thyroglobulin was dually labeled with [^{32}P] phosphate and [^{35}S]methionine or with [^{32}P]phosphate and [^{3}H]mannose, radioactivity measurements of the 330-kDa band excised from gel electrophoretograms indicated that tunicamycin caused a 50 to 60% inhibition of phosphate incorporation, an ~85% inhibition of mannose incorporation, but only a ~10% inhibition of methionine incorporation into the thyroglobulin secreted into the media (Table II). [^{3}H] Mannose incorporation into trichloroacetic acid precipitable media or cell proteins under the same conditions was 22 and

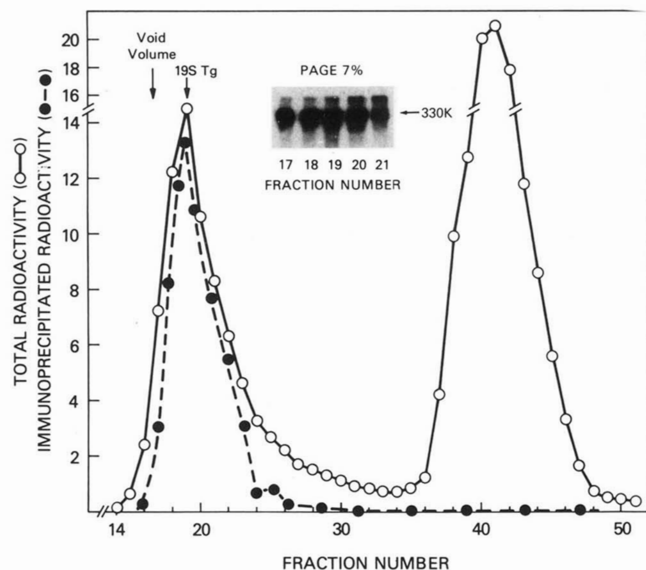

FIG. 2. **Sephacryl S-300 chromatography of an ammonium sulfate purified thyroglobulin preparation isolated from the media of rat FRTL-5 thyroid cells incubated with [^{32}P]phosphate.** The elution position of authentic 19 S rat or bovine thyroglobulin is noted by an *arrow* as is the position for free phosphate. ^{32}P radioactivity was measured on an aliquot of each fraction; a second aliquot was applied to 4% Laemmli gels (33) after denaturation in the presence of β-mercaptoethanol (33). Autoradiograms of gels from the noted fractions (*inset*) show that ^{32}P co-migrates with an authentic 330-kDa thyroglobulin marker. Excision of the 330-kDa band from the gel and direct measurement of the associated radioactivity indicated that ~99% of the recovered [^{32}P]phosphate was in the 300-kDa protein. *K*, kDa.

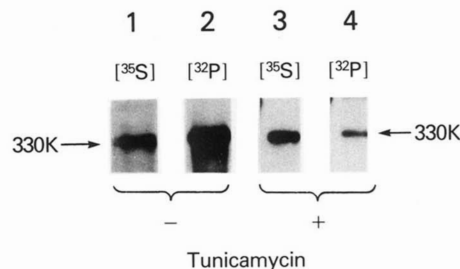

FIG. 3. **Gel electrophoretogram of [^{35}S]methionine- or [^{32}P] phosphate-labeled thyroglobulin immunoprecipitated from the media of FRTL-5 thyroid cells incubated with one or the other radiolabel in the absence (−) or presence (+) of 1 μg/ml tunicamycin.** "Materials and Methods" provides details of the radiolabeling incubation with or without tunicamycin. Before immunoprecipitation, the thyroglobulin was purified from the media by ammonium sulfate precipitation and Sepharose CL-6B gel filtration chromatography as in Fig. 1. Equal aliquots of thyroglobulin protein, measured by colorimetric analysis (44) and immunoassay (see "Materials and Methods"), were immunoprecipitated and the pellets applied to 4% Laemmli gels (33). Samples and gels were prepared and run under reducing conditions. Coomassie Blue staining confirmed that approximately equal amounts of protein were present in each 330-kDa band despite the differences in the autoradiograms. *K*, kDa.

36% of control values, respectively; [^{35}S]methionine incorporation was ~88% of control in both cases.

These data suggested that the phosphate residues were measurable in rat, human, or bovine follicular thyroglobulin, that they were covalently linked to the thyroglobulin, that rat FRTL-5 cells could be used to study the nature of these phosphate residues in thyroglobulin, and that some but not all of the phosphate might be associated with carbohydrate moieties on the thyroglobulin molecule.

Phosphate Residues on the B-Carbohydrate Moiety of Thyroglobulin

FRTL-5 Cell Thyroglobulin—To further define the nature of the phosphate residues, and in particular their potential association with carbohydrate moieties of thyroglobulin, FRTL-5 cells were radiolabeled with [^{32}P]phosphate and either [^{3}H]mannose or [^{35}S]methionine. The media thyroglobulin was isolated by immunoprecipitation; the immunoprecipitated pellet treated with Pronase; and the Pronase digests chromatographed on a Bio-Gel P-6 column to identify radiolabeled peptides (Fig. 4). Since Pronase can be argued to contain phosphatase activity, immunoprecipitation, Pronase digestion, and chromatography were performed in the presence of 150 mM phenyl phosphate in an attempt to minimize this problem.

As noted in Fig. 4, Pronase digestion of immunoprecipitated, dually radiolabeled thyroglobulin resulted in the formation of several major glycopeptide fractions wherein both the [^{3}H]mannose and [^{32}P]phosphate radiolabels co-migrated. Fractions 50–100, which contained these peptide fractions, were pooled, lyophilized, desalted, and subjected to endo-H or neuraminidase treatment (see "Materials and Methods").

As noted in Fig. 5*A*, the control incubation at pH 5.5 for 20 h at 37 °C did not significantly change the elution pattern by comparison to Fig. 4. In contrast, endo-H treatment under the same conditions (Fig. 5*B*) resulted in the release of a mannose-containing oligosaccharide, which also contained phosphate, from the dually labeled peptide fractions migrating between fractions 80 and 100 in Fig. 4 or 5*A*. Half of the phosphate-labeled, carbohydrate-containing peptides were, however, not endo-H-sensitive, *i.e.* those whose peak labeling was in or near fractions 66 and 73 (Fig. 4 or 5*A*). These two peptide fractions were, in contrast, neuraminidase-sensitive (Fig. 5*C*). Thus, neuraminidase treatment altered their elution from the column to regions between the endo-H-sensitive glycopeptide fractions (Fig. 5*C*).

Bovine Thyroglobulin—The above results could be confirmed using preparations of 19 S bovine thyroglobulin. Thus, when 500 mg of thyroglobulin were Pronase digested and chromatographed using conditions identical to those in Fig.

TABLE II

[^{32}P]Phosphate, [^{35}S]methionine, and [^{3}H]mannose radioactivity in the 330-kDa thyroglobulin bands excised from gel electrophoretograms of media thyroglobulin from FRTL-5 cells labeled in the presence or absence of tunicamycin (1 μg/ml)

FRTL-5 cell 19 S thyroglobulin secreted into the media was dually radiolabeled by incubating the cells with either [^{32}P]phosphate and [^{35}S]methionine or [^{32}P]phosphate and [^{3}H]mannose (see "Materials and Methods") in the presence or absence of 1 μg/ml tunicamycin. After ammonium sulfate purification and gel filtration over Sepharose CL-6B, the thyroglobulin was immunoprecipitated and subjected to gel electrophoresis. Areas of the gel co-migrating with the unlabeled 330-kDa thyroglobulin marker was excised and the radioactivity measured in a Beckman scintillation spectrometer. The amount of thyroglobulin protein was effectively identical in each pellet which was subject to gel analysis. Recovery of [^{35}S]methionine was 89 ± 4% in each case; recovery of the [^{32}P]phosphate was ~91 ± 3% in all experiments.

Tunicamycin	Radioactivity incorporated (cpm)			
	Experiment A		Experiment B	
	[^{32}P]Phosphate	[^{35}S]Methionine	[^{32}P]Phosphate	[^{3}H]Mannose
−	28,200	47,500	25,400	34,600
+	12,800	42,200	10,900	5,100
% inhibition by tunicamycin	55%	7%	57%	85%

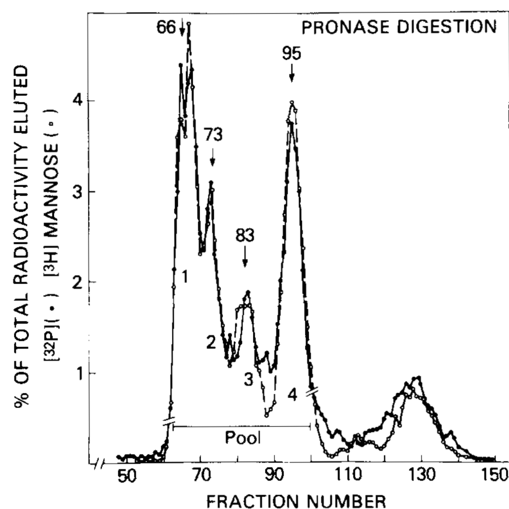

FIG. 4. **Elution pattern of Pronase-digested rat thyroglobulin from the media of FRTL-5 cells on a Bio-Gel P-6 (200–400 mesh) column (see "Materials and Methods").** The column was 186 × 1 cm; the fractions were 0.9 ml; and the flow rate was 10 ml/h. The FRTL-5 cell media thyroglobulin which was subjected to Pronase digestion had been dually labeled with [^{32}P]phosphate (●) and [^{3}H]mannose (○) by incubating cells as described under "Materials and Methods." The thyroglobulin was immunoprecipitated, subjected to Pronase digestion, and chromatographed in the presence of 150 mM phenyl phosphate as detailed under "Materials and Methods." Fractions were analyzed for their ^{32}P and [^{3}H]mannose content; fractions 50–100 were pooled, desalted, and lyophilized (see "Materials and Methods") in preparation for the experiment to be reported in Fig. 5.

4, two major glycopeptide fractions, noted (*a*) and (*b*), could be detected (Fig. 6). Both glycopeptide fractions contained coeluting phosphate residues which could be colorimetrically detected (Fig. 6*A*). Sugar analysis of peptide (*a*) indicated the presence of fucose, mannose, and galactose; sugar analysis of peptide (*b*) revealed only mannose (Fig. 6*B*). Endo-H treatment resulted in the coincident loss of glycopeptide (*b*) and its associated phosphate (Fig. 6*A*). Peptide (*a*) and its associated phosphate were not endo-H-sensitive.

In summation, these data using bovine thyroglobulin were effectively the same as presented for rat thyroglobulin. There appeared to be a group of phosphate residues associated with an endo-H-sensitive, mannose-rich glycopeptide, *i.e.* with the glycopeptide whose predominant carbohydrate moiety appeared to have characteristics of the B-carbohydrate moiety on thyroglobulin. Endo-H experiments indicated that this group of phosphate residues were covalently linked to the carbohydrate moiety itself. There was, however, a second group of phosphate residues on a glycopeptide whose predominant carbohydrate unit appeared to be of the A chain type, *i.e.* an endo-H-insensitive carbohydrate moiety containing galactose as well as mannose and neuraminic acid. There was no evidence, however, that there was a relationship between the A carbohydrate moiety and this second group of phosphate residues.

Nuclear Magnetic Resonance Studies Further Examine the Nature of the Heterogeneity

To provide some additional clues as to the nature of the different phosphate residues on bovine thyroglobulin, the preparation of 19 S follicular bovine thyroglobulin was examined by ^{31}P nuclear magnetic resonance. Fig. 7 shows the ^{31}P NMR spectrum of bovine thyroglobulin purified in the presence (*a*) or absence (*b*) of IMX. IMX is usually used as an inhibitor of cAMP phosphodiesterase activity but can

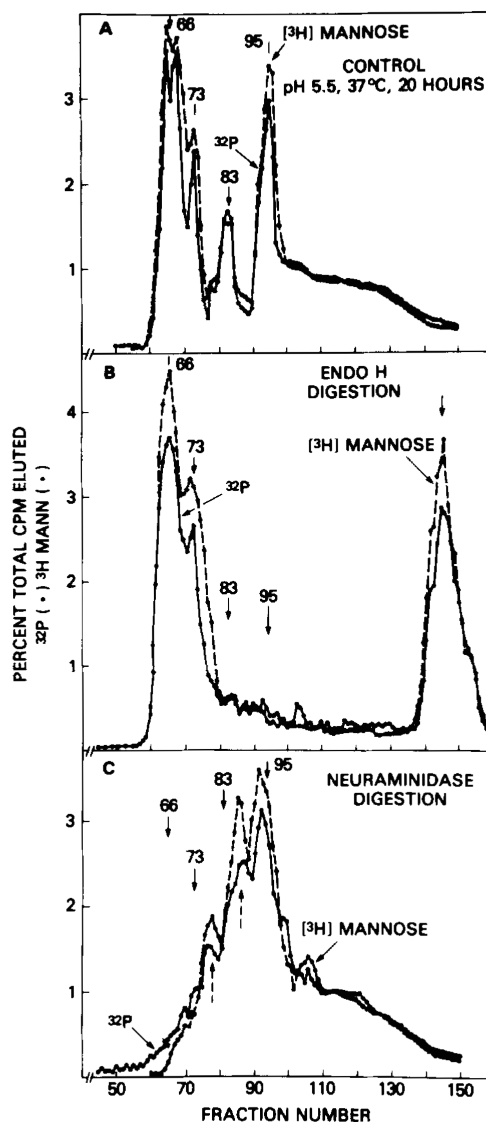

FIG. 5. **Rechromatography on a Bio-Gel P-6 column of the glycopeptide containing [^{3}H]mannose (○) and [^{32}P]phosphate (●) (fractions 50–100) from Fig. 4 after treatment with endo-H (*B*), with neuraminidase (*C*), or with no enzymes but subjected to identical conditions of incubation and rechromatography (*A*).** See "Materials and Methods" for details of pooling, desalting, and concentrating the noted fractions from Fig. 4 as well as the enzyme treatments. The column size, elution buffer, and elution rate were the same as in Fig. 4.

inhibit other enzymes capable of the hydrolysis of covalently bound phosphate residues. In the IMX-treated thyroglobulin (Fig. 7*a*), the ^{31}P spectrum showed three resonances centered at δ 1.5, 0.0, and −2.5 ppm. These resonances are typical, respectively, of ^{31}P resonances (i) in a number of sugar 6-phosphates and/or serine 0-phosphate (δ 1.5), (ii) in sugar 1-phosphate and/or inorganic phosphate (δ 0.0), and (iii) in phenyl phosphate, tyrosine-*O*-phosphate, or phosphodiesters such as present in pApA or the mannan core (δ −2.5). Since these resonances were not shown in the spectrum of the protein-free filtrate in the course of multiple sequential refiltration procedures, the data suggested that these phosphate residues were integral to the thyroglobulin molecule.

The relatively narrowed resonances (Δν ~25–30 Hz) at δ 1.5 and 0.0 ppm, which consisted of about 65 and 6% of the total phosphate signals, respectively, would be compatible with the existence of these phosphate groups in discrete

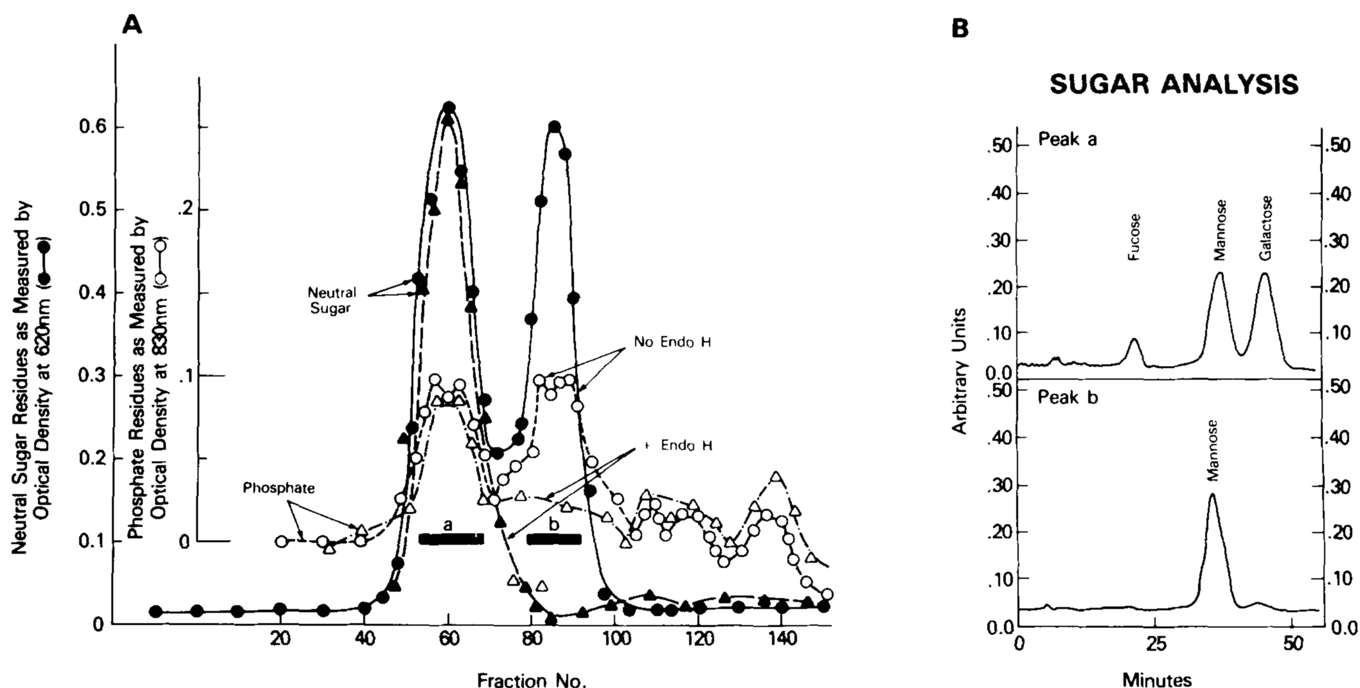

FIG. 6. *A*, chromatography on a Bio-Gel P-6 column of Pronase-digested bovine thyroglobulin before (O--O, ●--●) and after (Δ--Δ, ▲--▲) endo-H treatment. Fractions were analyzed for their content of neutral sugars (●, ▲) using the anthrone reaction (45) and absorption at 620 nm. Fractions were also analyzed for phosphate (O, Δ) using the Bartlett assay (40). Pronase digestion of 500 mg of thyroglobulin followed the same procedure as for the rat thyroglobulin (see "Materials and Methods" and Fig. 4). The column was 186 × 2.5 cm, equilibration was with Tris-chloride, pH 7.5, however *no* phenyl phosphate was in the equilibration buffer as was present in Fig. 4. This allowed colorimetric assays for phosphate except after fractions 140, where phenyl phosphate present during the Pronase digestion, began to elute. *B*, automated sugar analysis (46) of the two major glycopeptide fractions, denoted (*a*) and (*b*) in Fig. 6*A*, after pooling, desalting by rechromatography on Sephadex G-10 in distilled water, lyophilizing, and hydrolyzing as described (46). The identification of mannose was confirmed by the absence of any affect of glucose oxidase on the analyses (47).

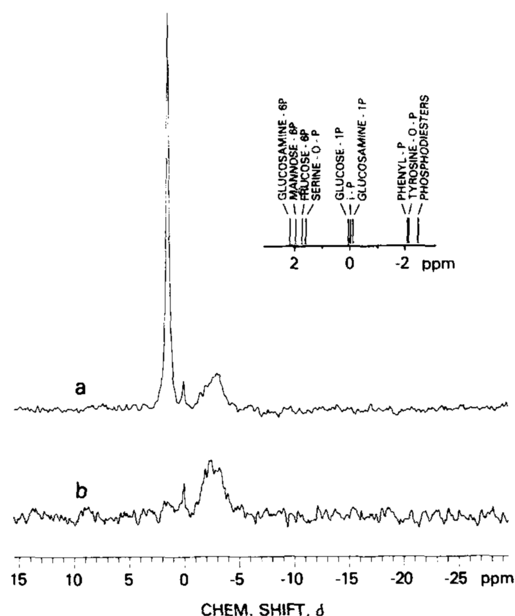

FIG. 7. **^{31}P nuclear magnetic resonance spectra of 19 S bovine thyroglobulin preparations purified in the presence (*a*) or absence (*b*) of IMX (see "Materials and Methods," as well as the text, for details).** The *inset* denotes the ^{31}P resonance of a number of phosphate-containing molecules as measured at pH 7.4 and under identical conditions.

moieties located near the surface of the protein and having motional freedom. The wide-spread signal ($\Delta\nu$ ~200 Hz) at δ −2.5 ppm, on the other hand, is indicative of heterogeneity existing within the remaining phosphate residues and/or the possibility that they experience some motional restriction.

For the bovine thyroglobulin purified in the absence of IMX, the ^{31}P NMR spectrum revealed only two resonances centered at δ 0.0, and −2.5 ppm (Fig. 7*b*). The loss of the resonance at δ 1.5 ppm in this thyroglobulin preparation, by comparison to the preparation purified in the presence of IMX (Fig. 7*a*), raised the possibility that this phosphate moiety had been modified or destroyed during the purification of the protein. To evaluate this possibility, we conducted the following experiment (Fig. 8). About 100 μl of the thyroglobulin preparation (~100 mg/ml), purified in the absence of IMX, was added to 500 μl of a 5 mM mannose 6-phosphate solution. The ^{31}P NMR spectrum of this solution exhibited a resonance at δ 1.5 ppm, typical for a sugar 6-phosphate linkage (Fig. 8*A*). With time, the intensity of this peak gradually decreased with the concomitant appearance of a new peak at about δ 0.0 ppm (Fig. 8*B*). After 3 days, the peak at δ 1.5 ppm nearly completely disappeared and the peak at δ 0.0 ppm gained its maximum intensity (Fig. 8*C*). The new peak was consistent with the presence of inorganic phosphate based on ^{31}P NMR spectra with proton decoupling and chemical analysis of filtrates after reconcentration in an Amicon apparatus. These results suggested that the δ 1.5 ppm signal might represent a sugar 6-phosphate residue whose presence or absence could be modified by an IMX-sensitive activity present in, or contaminating, different thyroglobulin preparations.

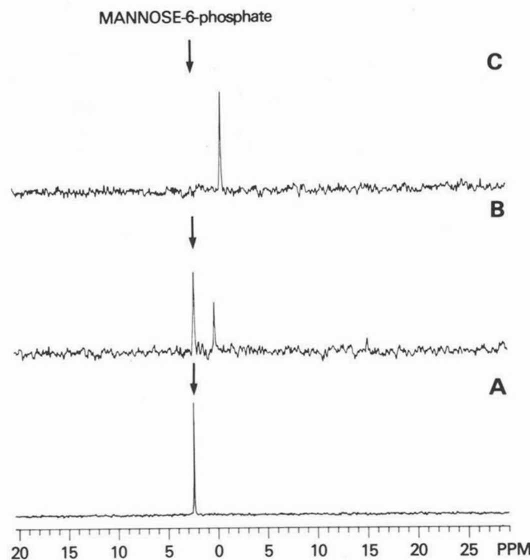

FIG. 8. **Ability of a thyroglobulin preparation prepared in the absence of IMX to modify the ^{31}P resonance of mannose 6-phosphate as a function of time of incubation.** A 100-μl aliquot of the thyroglobulin preparation (100 mg/ml) was added to a 5 mM solution of mannose 6-phosphate. ^{31}P NMR spectra were taken within the first hour after addition (*A*), after 24 h (*B*), and after 72 h (*C*).

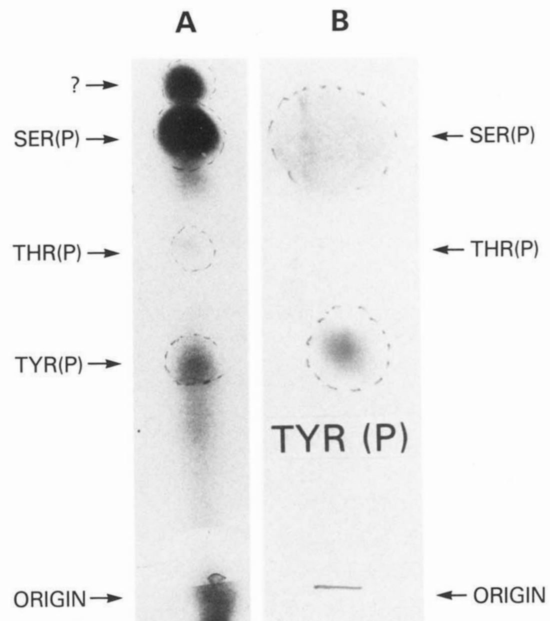

FIG. 9. **Phosphoamino acids present in ^{32}P-labeled thyroglobulin after purification by immunoprecipitation (*A*) and gel electrophoresis (*B*).** The labeled thyroglobulin from the FRTL-5 cell medium, purified by salting out and gel filtration chromatography (Fig. 2), was immunoprecipitated (see "Materials and Methods") and subjected to SDS-polyacrylamide gel electrophoresis. The 330-kDa band was identified by autoradiography, excised from the gel, electroeluted, lyophilized, and hydrolyzed with 6.0 N HCl as was the immunoprecipitate. The hydrolysates were separated by high voltage electrophoresis on thin layer cellulose plates (see "Materials and Methods"). *Dotted circles* indicated the position of the standard phosphoamino acids phosphoserine [*Ser(P)*], phosphothreonine [*Thr(P)*], and phosphotyrosine [*Tyr(P)*]. The standards, corun with the sample (see "Materials and Methods"), were detected by ninhydrin. [^{32}P]Phosphoamino acids and inorganic [^{32}P]phosphate were located by autoradiography.

Phosphoserine and Phosphoserine Residues in Thyroglobulin

The ^{32}P-labeled FRTL-5 cell thyroglobulin in the peak fractions of Fig. 2 (fractions 17–21) were pooled and divided into two equal portions. One portion was desalted, lyophilized, and subjected to phosphoamino acid analysis on thin layer plates after acid hydrolysis. The second portion was similarly treated but only after immunoprecipitation by a rat anti-thyroglobulin preparation and recovery as the 330-kDa subunits of thyroglobulin from SDS gels run under reducing conditions. It was evident that phosphotyrosine and phosphoserine residues were present in both thyroglobulin preparations (Fig. 9).

In the Pronase digestion studies described above (Figs. 4–6), approximately 50% of the phosphate residues were shown to be associated with a mannose-rich, endo-H-released oligosaccharide. Consistent with this observation, deglycosylation of the radiolabeled FRTL-5 thyroglobulin preparation resulted in the loss of 46% of the radiolabeled phosphate (Table III). Phosphoserine residues are very sensitive to alkali whereas phosphotyrosine residues are significantly less so (48–50). Alkali treatment of the radiolabeled FRTL-5 thyroglobulin preparation released ~30% of its radiolabeled phosphate; alkali treatment of deglycosylated radiolabeled FRTL-5 thyroglobulin released ~85% of its radiolabeled phosphate (Table III). The sum of these data suggested that thyroglobulin preparations contained ~10–12 phosphate residues (Table I and related text, Table III) of which ~5–6 were linked to the A chain oligosaccharide, ~3–4 were phosphoserine, and 2 were phosphotyrosine.

The presence of phosphotyrosine residues in rat FRTL-5 cell thyroglobulin was confirmed by immunoprecipitation of an [^{35}S]methionine-labeled preparation with an antibody directed against phosphotyrosine residues (Fig. 10). The phosphotyrosine antibody (Fig. 10, *lane 1*) precipitated a protein which on gel electrophoretograms migrated with a 330-kDa standard and in the same position as thyroglobulin immunoprecipitated from the same preparation by anti-thyroglobulin (Fig. 10, *lane 3*). A preimmune sera was not active in this regard (Fig. 10, *lane 2*) and a preincubation with the anti-thyroglobulin antisera removed the radiolabeled protein able to react with the anti-phosphotyrosine antisera preparation (data not shown). ^{125}I-labeled bovine thyroglobulin was also immunoprecipitated by the phosphotyrosine antibody (Fig. 10, *lane 6*) but not a preimmune sera (Fig. 10, *lane 5*).

The phosphoserine and phosphotyrosine residues appeared to be localized in a relatively discrete portion of the thyroglobulin molecule rather than being broadly distributed throughout the protein. Thus, after tryptic digestion of the radiolabeled FRTL-5 thyroglobulin and HPLC separation of the peptides, the radiolabeled phosphate was found in three areas (Fig. 11). After pooling of the three areas denoted *I*, *II*, and *III* in Fig. 11, they were lyophilized and subjected to acid hydrolysis and phosphoamino acid analysis on thin layer plates. Only pool III contained phosphoserine and phosphotyrosine (Fig. 11, *inset*). The sharp elution pattern of pool III suggested it was a relatively discrete peptide. Gel electrophoresis of this fraction indicated that ~80% of the phosphate was in a single peptide of molecular weight ~ 15,000. Approximately 68% of the phosphate in the peptide eluted from the gel was alkali-sensitive. In experiments wherein thyroglobulin dually labeled with [^{3}H]mannose and [^{32}P]phosphate were similarly subjected in tryptic digestion and HPLC analysis, no tritiated mannose was evident in peak III. Tritiated mannose was, however, present in peaks I and II.

A similarly discrete localization of the phosphotyrosine and phosphoserine was evident in cyanogen bromide cleavage experiments. Thus, after cyanogen bromide cleavage, analysis of the peptides revealed that the majority of the radioactivity, 78% as determined from sliced gels, was in an ~24-kDa

TABLE III

Effect of deglycosylation and treatment with alkali on the phosphate distribution in ^{32}P-radiolabeled thyroglobulin molecules purified from FRTL-5 cell medium

FRTL-5 cell thyroglobulin, dually labeled with [^{32}P]phosphate and [^{35}S]methionine, were purified from FRTL-5 cell medium by ammonium sulfate fractionation, gel filtration chromatography, immunoprecipitation, and 4% SDS-polyacrylamide gel electrophoresis. The radiolabeled 330,000-molecular weight protein was identified by autoradiography, excised from the gel, electroeluted overnight, lyophilized, and either deglycosylated or treated with alkali as detailed under "Materials and Methods." Residual radioactivity was estimated on the 20% trichloroacetic acid pellet resultant from each treatment. Control experiments included incubations under the same conditions but in absence of enzymes (Experiment A) or NaOH and enzymes (Experiment B) showed no significant phosphate (<0.1 mol of phosphate/mol of thyroglobulin) release by comparison to untreated preparations. All assays were performed in triplicate.

	Molar ratio phosphate/thyroglobulin[a]	Phosphate released
Experiment A		
Thyroglobulin (control)	12.0 ± 0.2	100
Deglycosylated thyroglobulin	6.5 ± 0.4	46
Experiment B		
Thyroglobulin (control)	11.0 ± 0.1	100
Alkali-treated thyroglobulin	7.8 ± 0.3	29
Alkali-treated deglycosylated thyroglobulin	1.8 ± 0.5	84

[a] The amount of phosphate in the FRTL-5 cell radiolabeled thyroglobulin was estimated from the phosphate mg atoms incorporated into the protein (see "Materials and Methods").

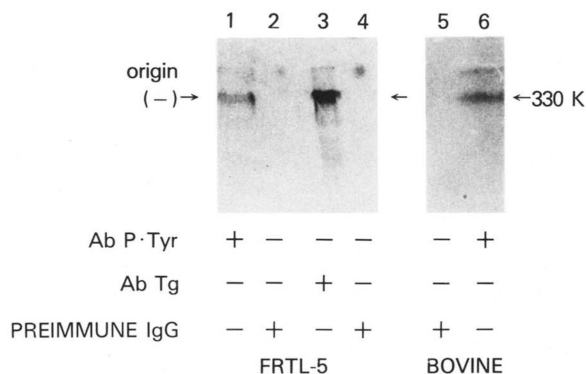

FIG. 10. **Immunoprecipitation of [^{35}S]methionine-labeled FRTL-5 cell or ^{125}I-labeled bovine thyroglobulin by an antisera directed against phosphotyrosine (*Ab P.Tyr*) residues (*lanes 1* and *6*, respectively).** In each case the immunoprecipitate was subjected to SDS-gel electrophoresis and autoradiography (see "Materials and Methods"). The [^{35}S]methionine-labeled FRTL-5 cell thyroglobulin immunoprecipitated by anti-rat thyroglobulin (*Ab Tg*) migrates in the same place on the gels (*lane 3*) as does the protein immunoprecipitated by the phosphotyrosine antibody (*lane 1*). Control, preimmune sera (*lanes 2, 4* and *5*) yielded negative results in all experiments. The antiserum in this experiment was kindly provided by Dr. C. R. Kahn; identical results were obtained using the antiserum provided kindly by Dr. P. Comaglio (39). *K*, kDa.

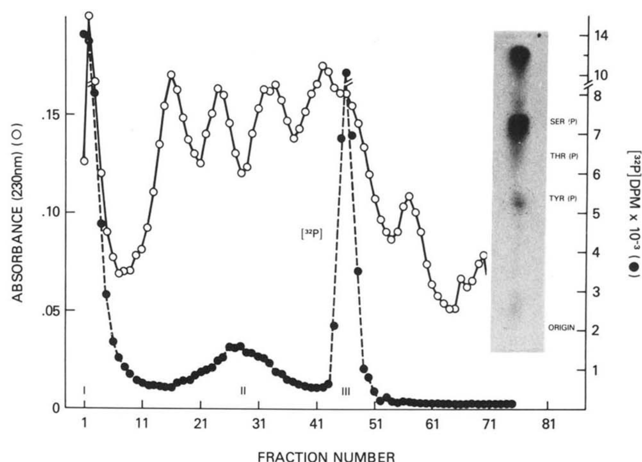

FIG. 11. **HPLC separation of ^{32}P-labeled phosphopeptides obtained by tryptic digestion of ^{32}P-labeled FRTL-5 cell thyroglobulin.** Purified ^{32}P-thyroglobulin from FRTL-5 cell medium (see legends to Figs. 2 and 9) was digested with trypsin as described under "Materials and Methods," and the [^{32}P]phosphopeptides separated by HPLC (see "Materials and Methods"). The *open circles* represent the absorbance at 230 nm of the cold rat thyroglobulin carrier; the *dark circles* represent the elution pattern of the ^{32}P radiolabel measured in each fraction. The *inset* depicts the phosphoamino acid analysis of the ^{32}P-labeled material in peak III, *i.e.* in fractions 44–48. The pooled peak III fractions were lyophilized; hydrolyzed, and the phosphoamino acids separated by high voltage electrophoresis as in Fig. 9.

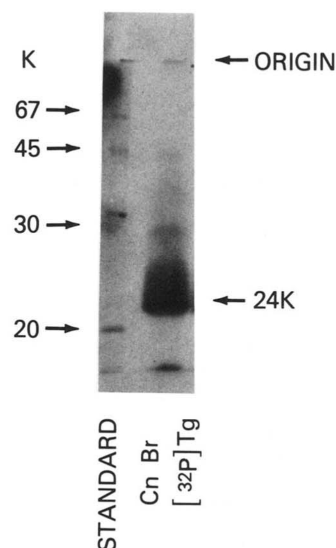

FIG. 12. **Autoradiography of ^{32}P-labeled cyanogen bromide peptides which were derived from [^{32}P]labeled FRTL-5 cell thyroglobulin and which had been subjected to SDS-polyacrylamide gel electrophoresis on 15% slab gels.** Cyanogen bromide (*CnBr*) treatment was as described under "Materials and Methods"; electrophoresis was under reducing conditions according to Laemmli (33). The *left lane* depicts the molecular weight standards; the *right lane* has the [^{32}P]phosphopeptides from the cyanogen bromide-treated thyroglobulin (*Tg*). *K*, kDa.

peptide (Fig. 12). This would not be expected to be carbohydrate-linked phosphate since prolonged (overnight) acid hydrolysis (70% formic acid) at room temperature (see "Materials and Methods"), the condition used for the cyanogen bromide cleavage procedure, would be anticipated to release the oligosaccharide-linked phosphate. This was confirmed in experiments using FRTL-5 cell thyroglobulin dually labeled with [^{3}H]mannose and [^{32}P]phosphate, *i.e.* the ~24-kDa cyanogen bromide peptide electroeluted from gels contained negligible amounts of tritium.

To see if these data were at all relevant to thyroglobulin preparations other than those isolated from the media of FRTL-5 cells, follicular 19 S bovine thyroglobulin was subjected to the same treatments as in Table III. Deglycosylation or acid treatment released ~50% of the phosphate residues on bovine thyroglobulin; 30–35% were alkali-sensitive; and

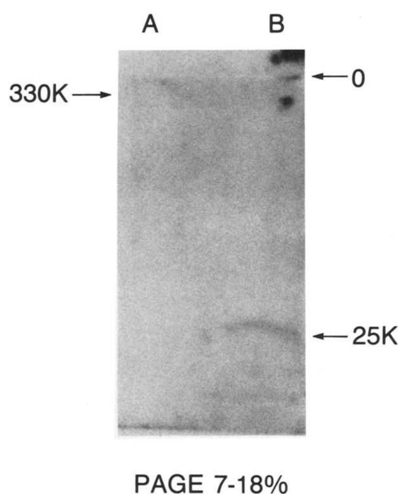

FIG. 13. **Detection of phosphotyrosine residues in a tryptic digest of bovine thyroglobulin using an antibody to phosphotyrosine residues.** Tryptic digested bovine thyroglobulin were subjected to SDS-polyacrylamide gel electrophoresis (7–18% gradient gel) and the protein transferred to a nitrocellulose sheet. The nitrocellulose sheets were incubated with an antibody to phosphotyrosine residues, (*lane B*) or its paired preimmune sera (*lane A*), the sheets washed, and ^{125}I-protein A added to detect any bound antibody. The conditions of trypsinization and of the immunoblotting are described under "Materials and Methods." Molecular weights were determined from simultaneously run labeled and unlabeled standards. *K*, kDa.

the residual phosphate residues were stable to both conditions as well as reactive with the phosphotyrosine antibody preparation after tryptic digestion and immunoblotting (Fig. 13).

DISCUSSION

The present report shows that human, rat, and bovine 19 S follicular thyroglobulin preparations, purified by standard procedures can contain ~10–12 mol of phosphate/mol of 660-kDa protein. The phosphate residues on thyroglobulin can be radiolabeled *in vitro* and are covalently associated with the thyroglobulin molecule. Thus, the phosphate is not removed by solvation in high salt concentrations (ammonium sulfate) by successive chromatography and gradient centrifugation procedures, by dialysis, even in urea or guanidine hydrochloride, nor by exposure to sodium dodecyl sulfate. The phosphate residues are associated with the thyroglobulin molecule even after it is converted to its 330-kDa subunit form after being subjected to SDS-gel electrophoresis under reducing conditions. The use of a rigorous array of purification procedures insures that the phosphate residues are not on contaminant proteins.

Evaluated using both rat and bovine preparations of thyroglobulin, the phosphate residues do not appear to represent a single homogenous population. Approximately one-half of the residues are present in endo-H-sensitive glycopeptide(s) whose carbohydrate moiety is of the mannose-rich, B chain type. The present data indicate that the phosphate residues on the glycopeptides containing the mannose-rich B carbohydrate moiety of thyroglobulin are covalently linked to the carbohydrate rather than the protein moiety. Thus endo-H treatment simultaneously released the phosphate as well as the mannose and both migrated on a gel filtration column as a single entity. These data are consistent with the observation that tunicamycin treatment of cells reduced the phosphate content in thyroglobulin by only approximately one-half, whereas at the same time causing an 85% reduction in mannose content. Also consistent are the data which show that 5–6 phosphate residues (~one-half of the total) are removed by enzymatic deglycosylation of the rat and bovine preparations. The ^{31}P NMR studies would suggest that in thyroglobulin preparations prepared in the absence of IMX, the carbohydrate-linked phosphate residues may exist in a phosphodiester linkage, whereas the carbohydrate linked thyroglobulin preparations prepared in the presence of IMX may include sugar 6-phosphate residues. This is evidenced by a predominant signal with a δ −2.5 ppm resonance in the former preparation, the presence of the δ 1.5 signal in the latter preparation, and the data of Fig. 9.

The phosphate residues associated with the peptides containing the neuraminidase-sensitive A carbohydrate moiety do not appear to be carbohydrate linked. Rather, the present report shows that 3 to 4 of the remaining phosphate residues are phosphoserine as evidenced by alkali sensitivity and 2 are phosphotyrosine. In FRTL-5 rat thyroid cell thyroglobulin, this is also evidenced by their presence in acid hydrolysates, their insensitivity to deglycoslyation, and/or their reactivity with an antibody to phosphotyrosine residues. These results do not appear to be unique to FRTL-5 cell thyroglobulin. Thus, nearly identical data concerning the number and heterogeneity of the phosphate residues with respect to sensitivity to alkali, insensitivity to deglycosylation, and reactivity with antibodies to phosphotyrosine residues were demonstrated using 19 S follicular thyroglobulin purified from bovine thyroid tissues. The phosphoserine and phosphotyrosine residues may be in a relatively discrete area of the protein based on their primary localization in what appears to be a single tryptic or cyanogen bromide peptide. The reason for the different size of the relevant tryptic peptide in FRTL-5 (15 kDa in Fig. 11 and related text) as opposed to bovine (25 kDa in Fig. 13) thyroglobulin preparations is unclear but in no way violates the presumption of a nonrandom distribution of these residues along the thyroglobulin molecule.

The role of phosphoserine or phosphotyrosine residues in thyroglobulin structure, synthesis, biology, or biochemistry is unclear as is the means of phosphorylation. Dog thyroid cells (51) and the rat FRTL-5 cells (52) do contain a protein kinase C system. FRTL-5 cells also contain insulin and IGF-I receptors with phosphorylating ability (53); insulin and IGF-I can increase thyroglobulin synthesis and message formation in the absence of thyrotropin (53–55). The size of the cyanogen bromide fragment, ~24 kDa, is compatible with either the C- or N-terminal peptide of thyroglobulin (56, 57), both of which are the preferential sites of thyroid hormone formation (41, 58, 59). The possibility is thus raised that they might be important in the process of iodination or in thyroid hormone formation; this point is, however, speculative and must await additional studies.

The role of the carbohydrate-linked residues is also unclear. In fibroblasts, sugar phosphate residues are linked to the vectoral transport of proteins to the lysosome and to lysosomal modification of those proteins (60–65). FRTL-5 rat thyroid cells and bovine thyroid membranes do have a mannose 6-phosphate binding site which can lead to degradation of thyroglobulin which is coupled with monophosphopenta-mannose *in vitro* (21, 24, 66). The relationship of the present data to those observations remains to be defined. Nevertheless, speculation exists (21, 24–27) that carbohydrate-linked phosphate residues on thyroglobulin could be linked to some way to the lysosomal degradation of thyroglobulin in an analogy to the fibroblast-glycohydrolase model.

Although the present report amplifies the results of the several recent reports (21, 24–27)[1,2] which showed that thyroglobulin contained phosphate residues, it is notable that several of the reports detected only one type of phosphate

residue and/or measured lesser amounts of phosphate than in this report. The data of Fig. 8 may, in part, explain the difficulty in measuring phosphate residues on thyroglobulin preparations. Thus, despite apparent homogeneity of the preparation, there appears to be a contaminating or associated phosphate hydrolyzing activity. The primary sequence of thyroglobulin and acetylcholinesterase has been shown to exhibit significant similarity (67); whether this observation is relevant to the enzymatic activity in the thyroglobulin preparations remains to be seen. Nevertheless, the use of phenyl phosphate or IMX in preparative buffers, in order to minimize this activity, should help in giving higher yields, particularly, perhaps, of the carbohydrate-linked phosphate residues.

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